

ON THE COVER

Cadmium yellow and cadmium red are two colours of paint featured prominently in the paintings of Impressionist and Post-Impressionist artists such as Claude Monet, Vincent Van Gogh, and Henri Matisse. Though we now take full-spectrum colour for granted, the brilliant, opaque shades of cadmium yellow did not become commercially available until after the Industrial Revolution in the 1840s, and cadmium red even later in 1919.

The chemical advancements of the Industrial Revolution allowed painters to produce their vibrant work, and in the present day, modern chemistry is equally important to preserve their lustre. The constituent chemicals of cadmium yellow and cadmium red, cadmium sulfide (CdS) and cadmium selenide (CdSe), are two of three cadmium compounds featured in this issue's work by Riddle et al. Their research (p11) uses Raman spectroscopy to examine these compounds' stability over a wide range of temperatures, motivated in part by their use in artwork past and present. The Raman spectra of CdS (courtesy of the author) is laid over top.

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FOREWORD

Dear Reader,

You hold in your hands the culmination of one decade of dedicated labour by the undergraduate Science community. From its humble beginnings at the 2005 Undergraduate Research Conference, the yearly publication of the McGill Science Undergraduate Research Journal has become a robust resource, giving students across disciplines and universities a voice for their research; uniting leaders and novices around the world as peer reviewer and researcher; providing undergraduates with invaluable critique of their writing style; and helping the uninitiated dive into the world of research through the hosting of workshops and the opening of invaluable dialogue regarding the world of academia.

As one of North America's first peer-reviewed undergraduate research journals, MSURJ is proud to have remained a bastion providing aspiring scientists a platform to communicate their findings and develop themselves as researchers. It is perhaps a testament to our core values that the authors of our inaugural volume have since become world-class researchers, some holding faculty positions at renowned institutions around the world.

These past ten years have proven to be years of tremendous growth and challenge at MSURJ. We couldn't have done any of it without the vivacious research culture at universities such as McGill; the contributions of our authors; the diligent work ethic of the editorial board; or the support of our advisors, our donors, and our readership.

On behalf of the editorial board of the tenth edition of MSURJ – we thank you.

Deborah Baremberg & Blair Jia
Co-Editors-in-Chief

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We thank all of our donors in the McGill community for their generous support:

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We extend our gratitude to the numerous peer reviewers who took the time to review all of the submissions.

Lastly, we wish to recognize the student contributors whose efforts have made the Journal possible.

Research Article

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Keywords

Cadmium chalcogenide: a cadmium(II) compound of a group VI element in the Periodic Table having the general formula CdX ($X = \text{S, Se, Te}$)

Raman spectroscopy: an important light-scattering technique named in recognition of the work of the Indian physicist, Sir Chandrasekhara Venkata Raman (1888–1970), who was awarded the Nobel Prize in Physics in 1930

II–VI semiconductor: a material comprised of two elements, one from group II and one from group VI in the Periodic Table, which has an electrical conductivity between that of a conductor such as copper and an insulator such as glass

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Cadmium(II) Chalcogenides Stable across Wide Temperature Range

Abstract

Background: The three cadmium(II) chalcogenides CdX ($X = \text{S, Se, Te}$; Cadmium with either Sulphur, Selenium, or Tellurium), have important applications as artists' pigments and in the electronics industry. The purpose of this study is to assess the structural stabilities of bulk, microcrystalline samples of the three cadmium(II) chalcogenides over a wide temperature range by examining the changes that occur in their Raman spectra.

Methods: We recorded the Raman spectra of the three cadmium(II) chalcogenides from -196°C to 500°C on a commercial instrument equipped with a microscope and a variable-temperature stage.

Results: While the Raman spectra of all three cadmium(II) chalcogenides exhibited significant peak shifts and broadening with increasing temperature, these effects were completely reversible. There is no evidence of phase transitions, indicating structural stability.

Conclusion: Taken together, our results show that the three cadmium(II) chalcogenides are resistant to structural changes throughout the almost -700°C temperature range investigated, thereby reinforcing their continued use in artwork and in the electronics industry.

Introduction

The three cadmium(II) chalcogenides, CdX ($X = \text{S, Se, Te}$), have wide ranging applications as artists' pigments (in the case of CdS and CdSe) and in the electronics industry (in the case of CdSe and CdTe). There are three polymorphs of cadmium sulfide, CdS . The hexagonal wurtzite polymorph of CdS is found in nature as the mineral Greenockite, which was named for the Englishman Lord Greenock, and is currently used as the artist's pigment cadmium yellow. (1) The industrial uses of the other two polymorphs of CdS are as photoresistors sensitive to visible and near-IR radiation and as thin films in the construction of solar cells. (2–4) Cadmium yellow has been commercially available since the 1840s and became fashionable as an artist's pigment because of its brilliant colour, light fastness and its prominence in artwork by such well-known artists as Monet, Van Gogh and Matisse. (5) Cadmium selenide, CdSe , became commercially available in 1919 as the artists' pigment cadmium red in the form of the stable wurtzite polymorph. It is nowadays usually blended with CdS to produce varying shades of yellow and red. (6) This material is also an important II–VI semiconductor in the electronics industry because it forms quantum dots with a tunable band gap in the visible region that depends on the nanoparticle size. (7) The third member of the chalcogenide series, cadmium telluride (CdTe), is a dark brown material that is not used at all as an artists' pigment due to its extreme toxicity when ingested, but it too is a well-established II–VI semiconductor. Colloidal CdTe nanoparticles form quantum dots used in gamma and X-ray detectors, while bulk CdTe is used to make photovoltaic arrays for thin solar panels that convert sunlight into DC electricity. (8, 9) When alloyed with mercury, CdTe is also used to manufacture HgCdTe (MCT) liquid N₂-cooled infrared detectors. (10)

Although the effect of varying temperatures on artists' pigments plays an

instrumental role in art conservation, little work has been done on the structural dependence of artists' pigments on temperature using modern spectroscopic techniques. (11–13) Previously, Raman spectroscopy has been used to monitor the phase transformation of the less stable zinc blende form of CdS nanoparticles into their wurtzite counterpart, following annealing in an argon atmosphere at $200\text{--}500^\circ\text{C}$ for 2 h. (14) Raman spectroscopy has also been used to study the formation of CdS pellets in the production of thin films by pulsed laser deposition, the doping of nanocrystalline CdS thin films with cobalt ions by ion implantation at 300°C , and the temperature dependence of CdS quantum dots from -258°C to room temperature. (15–17) Additionally, some conventional and micro-Raman spectroscopic data have been published on CdS nanoparticles following their heat treatment at 150°C for several hours. (18) In the case of cadmium red (CdSe), only a low-temperature (-253 to 27°C) Raman spectroscopic study has been reported for CdSe nanocrystals. (19) Similarly, only a brief analysis of the Raman spectra of bulk CdTe at room temperature, using 488.0- and 514.5-nm laser excitation, has been reported. (20, 21) In view of the somewhat limited vibrational spectroscopic studies at higher temperatures of the CdX ($X = \text{S, Se, Te}$) compounds in the bulk form, we decided to investigate the Raman spectra of these materials over a wide temperature range (-196 to 500°C). (22)

Materials and Methods

Microcrystalline CdX (X = S, Se, Te) samples were purchased from Alfa Aesar (Ward Hill, MA, USA) and were used as received. Variable-temperature micro-Raman spectra (5 accumulations, 5s exposure time) were recorded on an inVia Renishaw (Wotton-under-Edge, Gloucestershire, UK) microscope using a 514.5-nm argon-ion laser (16 mW, maximum power) or a 785-nm near-IR diode laser (~15 mW; absolute power 300 ± 30 mW; 0.05% power setting), a long working length 50X/0.75 objective and a Linkham (Tadworth, Surrey, UK) model THMS600 thermal stage fitted with a quartz window. Variable temperature measurements were performed chiefly in the -196 to 500 °C range at 50 °C intervals. The samples were allowed to equilibrate for 1 min at each temperature before recording the Raman spectra. Different gratings were used for the two lasers: 2400 grooves/mm (514.5 nm laser) and 1200 grooves/mm (785 nm laser). The spectral data were obtained and manipulated using the Renishaw wiRe2 proprietary software and the peak positions are considered to be accurate to at least ± 1 cm⁻¹.

Results and Discussion

The Raman spectrum of bulk, microcrystalline CdS exhibits two peaks at ambient temperature at 301 vs and 603s cm⁻¹, together with a much weaker peak at 906 cm⁻¹. The effect of increasing the temperature specifically on these two peaks from -160 to 500 °C is illustrated in Fig. 1.

The two main peaks have previously been attributed to the longitudinal

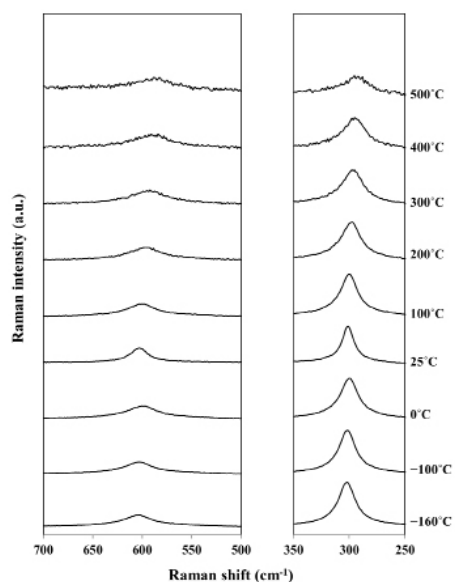


Figure 1. Variable-temperature (25 to 500 °C) spectra of bulk chrome yellow (CdS) pigment: changes observed for the 301 (1-LO) and 603 (2-LO) cm⁻¹ peaks.

optical 1-LO and 2-LO phonons, respectively (15, 18), while the 906 cm⁻¹ peak is presumably the 3-LO phonon or possibly a combination mode of the 1-LO and 2-LO phonons (calcd. 904 cm⁻¹). Moreover, as observed in the earlier low-temperature Raman study on CdS quantum dots, all three Raman peaks gradually shift to lower wavenumbers with increasing temperature. (18) In addition, the three peaks broaden considerably with increasing temperature [cf. the peak-width at half-maximum (PWHM) plot in Fig. 2] and a shoulder begins to appear on the low-energy side of the 1-LO phonon. This asymmetry has been noted previously in the Raman spectra of CdS nanoparticles and has been discussed in terms of the phonon confinement model. (16) The shapes of bands in the Raman spectra of solid materials are considerably affected by structural defects and the presence of these defects results in a violation of the Raman selection rules that leads to band broadening and asymmetry, as observed in our study

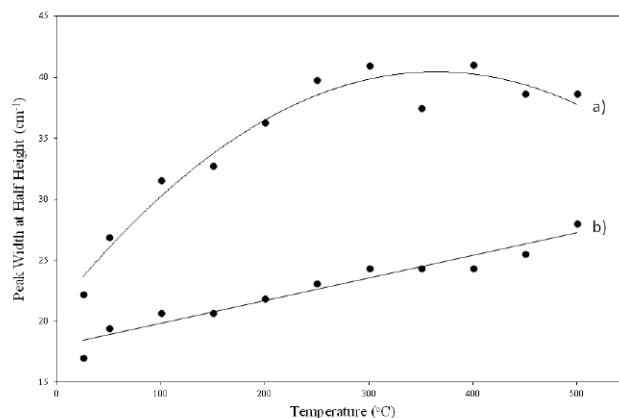


Figure 2. Temperature dependences of the peak widths at half maximum (PWHM) of the 301 (1-LO) and 603 (2-LO) cm⁻¹ modes of chrome yellow (CdS).

here. (23) The phonon confinement model is mathematical treatment of the shapes of Raman bands that takes into account these structural defects. A recent example of the use of this model is the analysis of the Raman band shapes associated with the LO phonons in III-V nanowires. (24) The quality of a material can be judged by a consideration of the so-called correlation length, which is the average size of the homogeneous regions of the material where there are no defects. The phonon confinement model has been almost exclusively applied to explain the asymmetry of LO Raman modes. The shift in the positions of the LO Raman bands is related to the size of the phonon confinement region in which a phonon can be restricted by such spatially limiting features as crystal twinning, stacking faults, vacancies, boundaries, and pores. (24) Considerable research has been reported on the analysis of defects in materials, including thin films, nanomaterials, and crystals using the phonon confinement model and there are now some excellent discussions on the topic. (25, 26) Upon cooling from 500 to -160 °C, the shifts and broadenings that were initially observed for the three phonons of CdS with increasing temperature are completely reversed.

Some similar Raman spectroscopic data were obtained in the variable-temperature (28-600 °C) Raman study of the bulk, microcrystalline cadmium red (CdSe) pigment. Three peaks are discernible at room temperature at 205, 413 and 618 cm⁻¹, which can once again be attributed to the longitudinal optical 1-LO, 2-LO and 3-LO phonons, respectively. These three peaks also shift to lower energies and broaden with increasing temperature. Above 200 °C, fluorescence dominates the Raman spectra, possibly because of a zinc blende to wurtzite phase change, as has been suggested earlier (27), but there is no clear evidence of such a phase transition. The spectral changes observed with increasing temperature are fully reversible upon cooling to ambient temperature.

In the case of cadmium telluride (CdTe), both the 514.5- and 785-nm lasers were used to obtain the Raman spectra. The latter wavelength was chosen to reduce the fluorescence associated with the previously-employed shorter laser excitation wavelength (488.0 nm) (20, 21). At room temperature with 785-nm excitation, five peaks are observed at 165.7, 330.2, 498.4, 664.9, and 841.2 cm⁻¹. The positions of the first three peaks agree quite well with those obtained at 223 °C using 488.0-nm laser excitation, and these peaks are assigned as the 1-LO, 2-LO and 3-LO phonons, respectively. (20,21) The two peaks located at 664.9 and 841.2 cm⁻¹ have not been observed previously and are attributed here to the 3-LO and 4-LO phonons, respectively, which may possibly have become active because of a resonance Raman effect. Interestingly, when the 514.5-nm laser is used, a completely different spectrum is obtained and a sharp doublet at 139.0 and 119.1 cm⁻¹ appears, together with a weaker broad peak at 744.0 cm⁻¹. There is also a shoulder evident at ~169 cm⁻¹, presumably the 1-LO phonon of CdTe. The sharp doublet at 139.0 and 119.1 cm⁻¹ is characteristic of optical modes of free elemental tellurium (Te), which may possibly have

been produced by laser irradiation of the CdTe surface. Elemental tellurium has been reported to exhibit Raman peaks at 139 and 120 cm⁻¹ and laser damage on the surface of thin CdTe films has been detected previously by Raman spectroscopy. (27, 28) The origin of the 744.0 cm⁻¹ peak is unclear, as it does not appear to be an overtone or a combination mode; it may, however, be associated with the formation of a cadmium or tellurium oxide species on the CdTe surface. (29-31)

Conclusion

The common features of the Raman spectra of the three cadmium(II) chalcogenides, CdX (X = S, Se, Te), are the broadening and shifting of the phonon peaks with increasing temperature. Both of these effects have been investigated recently for a number of systems, e.g., zirconia (ZrO₂) (29), the dye Nile Blue (33), the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (34), amorphous silicon (35) and Al₃BC₃ (36). Explanations have been put forward in the literature to rationalize these effects. One proposed explanation appeals to the dominance of anharmonicity at higher temperatures owing to the collapse of a temperature-independent force constant described by the harmonic oscillator model. Another, that photon decay causes the thermal expansion which results in shifts to lower wavenumbers of the vibrational modes and concomitant peak broadening. (37) All three compounds are quite resistant to structural changes throughout the wide temperature range investigated, which is an important characteristic that reinforces for their continued long-term use in artwork (CdS and CdSe) and in the electronics industry (CdSe and CdTe).

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Keywords

Greenhouse gases (GHG): an atmospheric heating phenomenon, caused by short-wave solar radiation being readily transmitted inward through the earth's atmosphere

Outgoing longwave radiation (OLR): the energy leaving the earth to space as infrared radiation

Stratosphere: the layer of the upper atmosphere extending upward from the tropopause to about 50 km above the earth, characterized by increasing temperature with altitude

Troposphere: lowest layer of the atmosphere, characterized by a steady drop in temperature with increasing altitude and is where nearly all cloud formations occur

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Earth Infrared Radiation Spectra During Global Warming Hiatus

Abstract

Background: Since 1997–98, observations of annual mean surface temperature have shown a slowdown of global temperature increases, suggesting a hiatus in global warming. Given this finding, we are interested in diagnosing trends in the Earth's outgoing longwave radiation (OLR) spectrum throughout the last decade.

Methods: We calculated the trend in OLR measured by the Atmospheric Infrared Sounder (AIRS) aboard NASA's Aqua satellite between 2003 and 2013, and compared these results with the trend in atmospheric and surface temperature and tropospheric absolute humidity, obtained from AIRS retrieval product and from the ECMWF (European Center for Medium range Weather Forecasting) Re-Analysis (ERA) interim product. We also isolated the greenhouse effect from the OLR trend by subtracting the amount of surface radiation emitted from the total radiation received by the sounder.

Results: The OLR trend is negative in the CO₂ absorption band, negative in the window spectral region, and positive in the water vapor band. The trend in surface and tropospheric temperature is negative, as is the trend in tropospheric absolute humidity. The greenhouse effect is increasing in the CO₂ band, generally slightly increasing in the window region, and decreasing in the H₂O band.

Conclusion: Our results show that the CO₂ forcing was still present globally through the last decade, with steadily increasing effects. Contributors to the negative trend in OLR in the window region are a small decrease in surface temperature and a strong decrease in tropospheric temperature, where tropospheric H₂O emit radiation to space. The decreasing effect of water vapor in the H₂O band is due to decreasing tropospheric humidity. This analysis will allow us to detect the changes in greenhouse gas forcing, to examine the correlated surface temperature response, and to study changes and effects in tropospheric water vapor concentration.

Introduction

Hiatus in global warming

Global surface temperature has increased the last century. This increase is a result of an increase in greenhouse gases concentrations, which causes an imbalance between ingoing and outgoing radiation at the top of the atmosphere (TOA). Currently, there exists an energy imbalance at the TOA, estimated to be 0.5–1 Wm⁻² over the 2000s. (1–6) There has been a hiatus in global warming – that is to say, a period of no or negative trend in surface temperature – over the past 15 years, triggering discussions about climate change. This phenomenon is not unique; global surface temperature has in the past exhibited periods of neutral or negative trends amid a longer period characterized by warming. (7) Hunt et al. (2011) have demonstrated that decade-long episodes of subaverage surface temperature can be sustained solely by internal variability, i.e. without external forcing, showing that the hiatus in global warming can be explained by a natural decadal cooling period. (8) Others argue that these decades of hiatus in warming result from stronger deep oceans heat uptake. (4, 6, 9, 10) It is also possible that the observed decrease in stratospheric water vapor concentration after 2000 has contributed to the cooling of the troposphere in the last decade. (11) Solomon et al. (2011) suggest that the observed increase in stratospheric aerosols in the 2010s have partly canceled out the radiative forcing from greenhouse effect by scattering solar radiation back to space. (12) To get a clearer picture of the true cause behind atmospheric changes, we

look to outgoing longwave radiation (OLR) spectrum of the past decade, which provides information about both the infrared radiation emitted by the earth's surface and the composition of the atmosphere.

Spectrum analysis of the outgoing longwave radiationshuang

A change in OLR can be caused by many factors, including changes in temperature at surface or gas emission levels, or changes in specific greenhouse gas concentrations. (13, 14) Earth's surface emitted infrared radiation (wavenumbers 0 to 3000 cm⁻¹) is proportional to the planet's temperature. As upwelling radiation crosses the atmosphere, it is absorbed by atmospheric particles and molecules that in return re-emit energy at specific wavelengths as a function of their temperature. If a greenhouse gas layer in the atmosphere increases in density and width, the layer becomes more opaque to infrared radiations coming from Earth's surface and consequently re-emits energy from higher, colder levels in the atmosphere. According to Stefan-Boltzmann's law, a colder greenhouse gas layer emits less radiation. Thus, the energy emitted to space decreases, resulting in a temporary imbalance between the incoming radiation from the sun and heat emission transmitted from the atmosphere to outer space. This can result in Earth's surface warming, increasing the upwelling flux of energy until the planetary energy balance is restored.

In order to analyze changes in atmospheric composition and in tropospheric and surface temperatures, we investigated spectral trends in

Earth's OLR. We studied changes in radiation in the CO₂ absorption bands ranging between wavenumbers 580 to 750 cm⁻¹ and 2200 to 2400 cm⁻¹. The spectral regions where the absorption of infrared radiation by CO₂ in the troposphere is the strongest are at wavenumbers 600 to 640 and 690 to 800 cm⁻¹. The radiative cooling of CO₂ in stratosphere is dominated by the 640-690 cm⁻¹ band. (8, 9)

In order to analyze the effects of changes in tropospheric humidity and in surface temperature, we studied the radiation trend of the water vapor absorption bands (wavenumbers 1400 to 1800 cm⁻¹) as well as in the OLR spectrum in the window region (wavenumbers 800 to 1300 cm⁻¹). In parallel with our analysis of the OLR trend, we analysed the trends in tropospheric and surface temperature and in tropospheric humidity through the same decade. We also isolated the effect of greenhouse gases in the OLR spectrum trend.

Methods

Data Download

We analyzed OLR data collected by the Atmospheric Infrared Sounder (AIRS) aboard satellite Aqua part of the NASA Earth Observing System. (17) Aqua has a polar sun-synchronous orbit and a repeat cycle period of 233 orbits (16 days). Its AIRS instrument is a continuously operating cross-track scanning sounder. We also retrieved calibrated and geolocated radiance data collected by the AIRS Version 5 Level 1B Infrared Radiance Products (AIR1BRAD).

We also analyzed tropospheric temperature and humidity data collected by Aqua Level 2 retrieval product (AIRX2RET) to be put in parallel with our previous results. (18)

The surface temperature used in our calculations of the temperature profile trend and in the greenhouse effect calculation was downloaded from the ERA-interim reanalysis product of the European Center for Medium range Weather Forecasting (ECMWF). (19)

All collected data represented the same time period: January 1st, 2003 to December 31st, 2013.

Data Processing

We averaged daily OLR measurements to calculate the 16-day mean. We then spatially averaged the data set over the world. We converted the radiances to equivalent brightness temperatures using the Planck function, the relationship between the radiance emitted by a body and its temperature. After, we filtered the 16-day mean data by deleting all wavenumbers at which the time series had more than 10 missing data points, which corresponds to less than 5% of the length of the time series. We then de-noised the 16-day mean of brightness temperature measurements. To do so, we set the maximum noise level to the 25th and 75th percentile of a normal function with mean 0 and variance 0.5, with precision 10⁻⁹. We removed spectra with points at one maximum noise level away from the 25th or the 75th percentile. Then, the de-noised 16 day mean data were averaged over years 2003 to 2013.

A similar method of processing was used to analyze the geophysical variables from AIRX2RET and ERA-interim.

Trend Calculation

To calculate the trend and standard deviation in observed OLR spectrum from the yearly mean data, we used the AR(1) method. (20) This method of calculation assumes that noise is autoregressive of the order of 1 (AR(1)). The confidence level is 95%.

We used the same method to analyze the geophysical variables from AIRX2RET, ERA-interim and simulated OLR spectrum.

Greenhouse Effect Calculation

We defined the greenhouse effect as the difference between the upward longwave radiation at the surface and the outgoing longwave radiation at the TOA. To calculate the greenhouse effect, we subtracted the annual

mean OLR spectrum from the annual mean surface radiation spectrum. The mean surface radiation spectrum was computed using the ERA-interim surface temperature and the Planck function, assuming that Earth is a black body. We then calculated the trend in greenhouse effect using the AR(1) method.

Results

In Fig. 1, we see the trend in OLR spectrum from 2003 to 2013 over the world. There is a negative trend in radiation in the CO₂ absorption bands. This reduced emission is signatory of increasing CO₂ concentration. (13, 15, 21) In fact, increasing concentration of CO₂ causes a rise in altitude of the levels of effective emission of CO₂ to space as well as emission of thermal infrared radiation. Because temperature decreases with height and radiation emissions is a function of the body temperature, the OLR is decreased. Therefore, the decreasing trend in equivalent brightness temperature at the CO₂ bands suggests that the CO₂ concentrations have been increasing in the last decade.

To compensate for this loss of energy to space, we expect the thermal infrared radiation from Earth to increase – in other words, a global warming in terms of surface temperatures. We can study surface emissions by looking at the OLR over the window region (wavenumbers 800 to 1300 cm⁻¹). At these wavenumbers, the changes in signal are dominated by the changes in surface temperature and are also affected by the changes in lower troposphere humidity in clouds and in aerosols. The results in Fig. 1 show a negative trend in equivalent brightness temperature over the window region. If it is caused by a decreasing surface temperature, then the data are consistent with the observed hiatus in global warming. (7)

The H₂O absorption band (wavenumbers 1400 to 1600 cm⁻¹) exhibits a positive trend in radiation. For equivalent brightness temperature to increase at these wavenumbers, the concentration in water vapor has to decrease such that the level of emission of water vapor is at lower altitude and warmer level. This positive trend could be also caused by increasing temperatures at the level of effective emission of water vapor.

Fig. 2 presents the trend in surface and atmospheric temperatures and in tropospheric humidity at different pressure levels (atmospheric pressure is here used as a measure of altitude) for the same time period. Note that pressure at the tropopause is around 200-100 hPa. The trend in surface temperature less than one standard deviation away from zero, which is consistent with the hiatus in global warming reported by the literature. (7) However, the trend in the lower tropospheric temperature (900-1000 hPa) is significantly above zero (more than one standard deviation away from zero). Part of this difference in trends can be a result of sampling biases (given the fact that the geographic region and altitude covered by a surface temperature sample will not be the same as, by example, a 1000 hPa level sample) – however, the 1000-900 hPa trend is consistent with the trend at the next higher level. Therefore, the trends in atmospheric temperature calculated from AIRS is ambiguous.

The slightly negative trend in surface temperature in Fig. 2.a is consistent with the negative trend in OLR over the window region (see Fig. 1), but is too weak to completely explain the decrease in OLR. The negative trend in tropospheric water vapor in Fig. 2.b is in agreement with the positive trend in OLR over the H₂O absorption band. Because tropospheric temperature is strongly decreasing, this last observation implies that in the H₂O band, the change in tropospheric water vapor concentration had more impact than tropospheric temperature change on the OLR trend in the H₂O band. Moreover, a decrease in tropospheric humidity causes an increase in OLR not only in the water vapor band but also in the window region (water vapor continuum). (14) However, OLR in the window region is decreasing, so there should exist a stronger contributor that cancels this effect from water vapor. We suspect the important decreasing tropospheric temperature to cause cooling at altitudes at which water vapor emit radiation to space, provoking a decrease in OLR. These results suggest that a decrease in tropospheric temperature contributes to the negative trend in OLR in the window region (Fig. 1).

Greenhouse Effect

By isolating the greenhouse effect (GHE) from the surface radiation in the OLR spectrum, we can identify more clearly the contributors to the change in OLR, especially in the window region. In Fig. 3, we compare the average OLR spectrum with the average surface radiation over the world from 2003 to 2012. The difference between the two spectra is mainly due to the GHE. The CO_2 , O_3 , and H_2O absorption bands are clearly noticeable at the 650-800, 980-1080 and 1400-1600 cm^{-1} spectral ranges, respectively, where the OLR spectrum is particularly lower than the surface upward IR spectrum.

Fig. 4 presents the trend in the greenhouse effect from 2003 to 2012. The greenhouse effect increased in the CO_2 band (wavenumbers 650 to 800 cm^{-1}) and decreased in the water vapor band (wavenumbers 1400 to 1600 cm^{-1}). These trends in the GHE could be caused by changes in greenhouse gas concentration or by changes in temperature at the level of the gas emission. There are also several significantly positive trends, namely in GHE over the window region (wavenumbers 800 to 1300 cm^{-1}), and at the O_3 band (at the center of the window region). These positive trends in GHE in the window region suggest that water vapor has increasing greenhouse effect in this spectral range.

Discussion

In this study, we analyze the trend in observed Earth thermal infrared radiation between 2003 and 2013 over the world, in particular through the window region, as well as the H_2O and CO_2 absorption bands.

In the CO_2 absorption band of the OLR spectrum, we are able to observe an evident decreasing trend in radiation. Moreover, following the greenhouse effect, there was increasing GHE over the CO_2 band. These results imply that the trend in CO_2 forcing was present worldwide during the hiatus in global warming of the last decade. According to literature, the OLR in the CO_2 band was also decreasing in the previous decades. (15, 16)

We also looked at the trend in equivalent brightness temperature (emission temperature) in the window region, where the emission is dominated by changes in surface temperatures, but also affected by water vapor concentration and clouds. We compared the observed trend in radiation with the observed trends in surface temperature, tropospheric temperature, and humidity to determine which one could be responsible for the change in radiation. Globally, from 2003 to 2013, there was a small negative trend in OLR through the window region. We propose that the main contributors to this reduced emission were a slight negative trend in surface temperature and changes in water vapor emission, particularly due to cooling at altitudes at which H_2O emits to space. In previous decades, the trend in OLR in the window region was positive (11,12).

In the water vapor absorption band, the trend in OLR over the world between 2003 and 2013 is positive. Coupling this observation with our other results, such as the negative trend in greenhouse effect at the same wavelength and the decrease in tropospheric humidity, we can conclude that humidity decreased globally in the last decade, along with its effect in the water vapor absorption band. Moreover, considering that tropospheric temperature was mainly decreasing, we can propose that changes in gas concentration had more effect on the OLR trend than did change in temperature of the layer of emission.

It would be relevant to use global climate model simulations to further identify key atmospheric contributors to this global cooling of the lower troposphere and clarify the impacts of the change in humidity on these observed trends in the OLR spectrum. This study does not take into account cloud cover, which can be addressed in future studies. In fact, cloud cover can mask OLR emitted by the surface below and emit OLR at higher altitude, further lowering measured temperatures. As a result, in cloud-covered skies, the OLR measured in the window region is a function of the clouds temperature instead of the surface temperature, which biases our analysis.

In the broader context of climate change, this study shows that although if

global warming has paused in the last decade, the greenhouse effect of CO_2 is still increasing. Therefore, the source of the problem of climate change is not solved. Considering that this hiatus in global warming is caused by a natural decadal cooling period, we expect that the global warming will resume with renewed strength in the next decade.

Figures

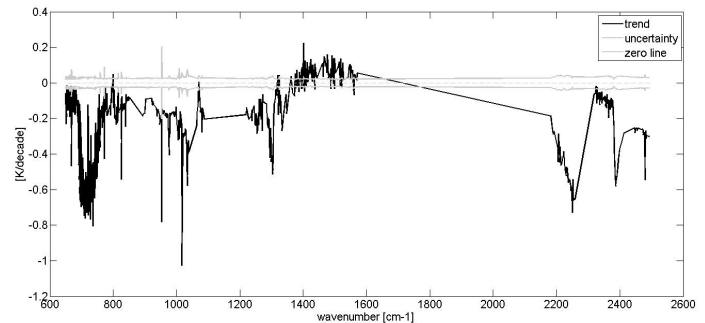


Figure 1. Trend in equivalent brightness temperature measured from OLR at different wavenumber from 2003 to 2013 over the global Earth (all latitudes and all longitudes). The spectrum was taken from 650 cm^{-1} to 2500 cm^{-1} .

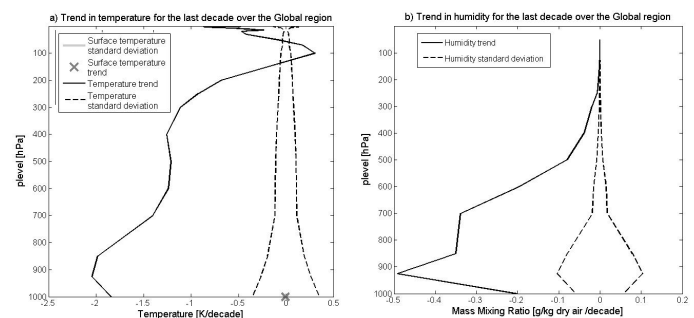


Figure 2. Trend in a) temperature and in b) humidity in function of level (1000 to 0.1 hPa) from 2003 to 2013 over global Earth.

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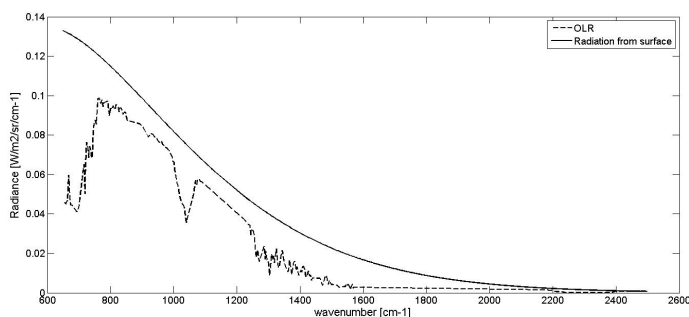


Figure 3. Comparison between climatology of OLR spectrum and surface radiation spectrum from 2003 to 2012 over global Earth. The spectrum was taken from 650 cm⁻¹ to 2500 cm⁻¹.

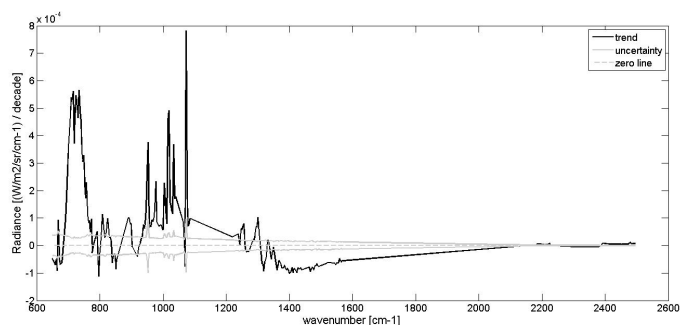


Figure 4. Trend in greenhouse effect trough longwave spectrum, from 2003 to 2012 over global Earth. The spectrum was taken from 650 cm⁻¹ to 2500 cm⁻¹.

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Keywords

Digital holography: a numerical reconstruction of a scene captured through interferometry, which presents multiple perspective of the scene in 3-D space

Mach-Zehnder interferometer: a device that extracts spatial information from an object using two split beams, whose phase-shift after reflection reveals the depth from a light source to the object

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Optimisation of a Holographic Microscope for Microbial Study

Abstract

Background: Tracking of microbial organisms over a volume requires images at multiple focal planes along the orthogonal direction. With most conventional microscopes, this requires repeated readjustments; using Digital Holographic Microscopy (DHM), it is possible to use a set of interference patterns to reconstruct at various distances, thereby creating a 3D stack based off a single image.

Methods: We used an off-axis Mach-Zehnder DHM for imaging and tracking bacterial movement. We describe the algorithm employed for tracking, as well as our improvement of trackability by testing differences in image contrast with the use of Quantum Dots.

Results: We show that the use of Quantum Dots resulted in an increase in contrast of approximately 11%.

Conclusion: We suggest this as a method of increasing resolvability of individual microbes. With a more compact design, the microscope will be applicable in various fields, and can be used remotely for studies of microbial organisms.

Introduction

Digital Holographic Microscopy (DHM) implementations use coherent interference between a clear “reference” beam and an “object” beam transmitted through, or reflected by, a sample of interest. This encodes the complex wavefront (amplitude and phase) as intensity modulations (fringes) on the detector plane. (1-4) Following this physical recording, the reconstruction process can be undertaken on a computer as a post-processing step. The encoded object wavefront of interest is retrieved from the digital hologram, and can be numerically propagated to any plane of interest using Fresnel Algorithms or Rayleigh-Sommerfield diffraction formula. (5-8) Finally, the amplitude and phase components of the complex wavefront in the target plane are computed to be saved for later analysis. The post-processing propagation procedure can be run repeatedly with different propagation distances to build a 3-D stack from a singly acquired hologram for each timeframe, without the need to scan in depth. .

Instrument & Samples

Microscope Configuration

The microscope used was a dual-beam off-axis Mach-Zehnder (MZ) type of DHM configuration, using a magnifying lens to reach the targeted sub-micron lateral resolution required for bacterial imaging (Fig. 1).

A blue $\lambda = 405$ nm laser was used as the coherent light source. The camera used was a 2448 x 2050 CCD with 3.45 μm pixel size, delivering up to 22 fps in 1024 x 1024 (1 Mpx) mode and 14 fps with 2048 x 2048 pixels (4 Mpx). Using a Thorlabs Geltech[®] aspheric lens (focal length = 8 mm and NA of 0.5) as a magnification $M = 20\times$ single-lens objective, we achieved a design field-of-view (FOV) of about 0.35 x 0.35 mm². For high-speed hologram reconstruction, we employed the LynceeTec Koala[®] off-axis DHM reconstruction software. (9)

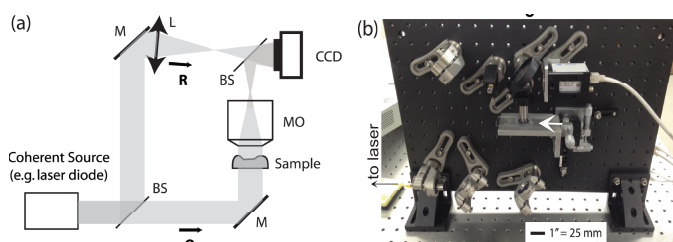


Figure 1. Instrument design. (a) General schematic showing a typical Mach-Zehnder off-axis DHM scheme. Legend: O, Object beam; R, Reference beam; BS, Beamsplitter; M, Mirrors; L, Lens; MO, Microscope Objective. (b) Photo of the actual breadboard instrument, with its fibered laser diode source injected in the bottom left.

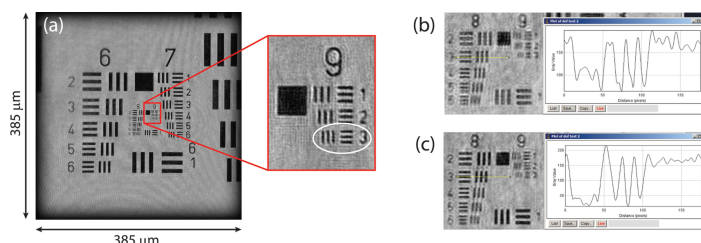


Figure 2. MZ-DHM optical performances assessment with a USAF test target sample. (a) Intensity-reconstruction image, with the inset zooming on the highest resolution elements: the white circle indicates 0.78 μm -wide scale bars; (b,c) Zoomed images on the smallest elements (groups 8 and 9) of (a), but reconstructed for the target moved along the optical axis (focus) by -1 mm (b) or +1 mm (c) away from the nominal working distance used in (a): the 3rd element of group 8 (1.55 μm -wide) is still well resolved in both cases as shown in the cross-profiles, indicating less than a loss of less than factor two in resolution vs. the nominal 0.8 μm .

Using a NIST-compliant high-resolution USAF test target as a sample (Fig. 2), we measured a lateral FOV of $0.385 \times 0.385 \text{ mm}^2$ over 1980×1980 pixels indicative of an effective magnification of about 18x (Fig. 2a). The target was also vertically shifted from the nominal focal height and the contrast ratio of the target elements were compared to receive a depth of focus in z (Fig. 2b, 2c). Overall, we have a volume of investigation of $0.38 \times 0.38 \times 2 \text{ mm}^3$, and can maintain nominal $< 0.8 \text{ mm}$ resolution inside $0.38 \times 0.38 \times 0.6 \text{ mm}^3$.

Biosamples

Samples at realistic densities could be imaged without filtration or dilution. To study environmental bacteria on the order of $1 \mu\text{m}$ in their largest dimension, pure cultures were imaged in optimal conditions. Fig. 3 shows phase and intensity images of three test strains: (a) *E. coli*, (b) *V. alginolyticus*, and (c) *B. subtilis*. The phase images showed slightly more contrast and were more readily used for tracking. Although the individual bacteria were difficult to identify in still images, their motion was very clear in real time. *B. subtilis* spores (mean length, $1.1 \pm 0.1 \mu\text{m}$; width, $0.48 \pm 0.04 \mu\text{m}$) could be readily resolved in phase (Fig. 3c inset). (11)

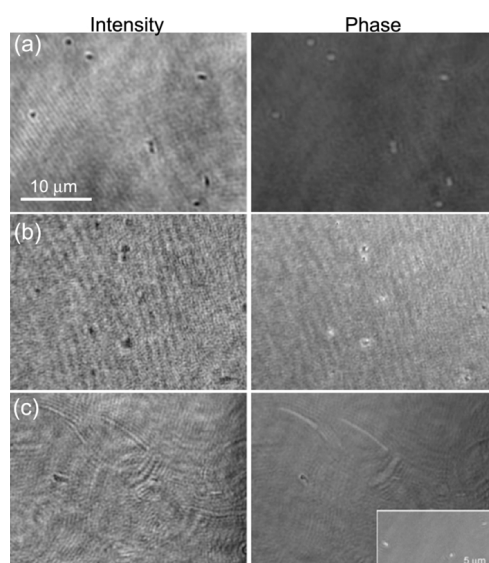


Figure 3. Images of bacteria amplitude (left) and phase (right). Cell densities represent approximate optical densities of 0.05 (organisms per pixel) for (A) *E. coli*, (B) *V. alginolyticus*, (C) *B. subtilis* vegetative cells and (inset) spores.

Bug Extraction

Blob Finding

For tracking, the software must be able to autonomously locate the organisms. While various programs were tested for this purpose, the algorithms employed by most programs are similar and can be done using Matlab. (10) Using the image processing tools, we averaged dynamic range over the whole stack for phase images. This enabled a constant mean value and removed image flicker over time. Then for each time-step, we increased contrast throughout, thresholded, and used background separation to locate individual bug cells as blobs. Using cell count, we recorded properties of each blob. This gave the dimensions and location shown in Fig. 4. All steps were repeated for the next time-step.

While the process worked very well for objects that were clearly distinct from the background, success rate with bugs was very low. For a sample bacterial image, false positives (non-bugs captured) was 86%, while false

negatives (bugs not captured) was 69%, based on visual inspections. This led to our conclusion that improvement in contrast was necessary for better tracking results.

The results may differ slightly with volume, as each image generally contains traces of bugs along out-of-focus planes. In fact, bug accumulation over z causes further loss of contrast. To visualize bug accumulation in a region, overall bug paths can be generated for individual 2D stacks by integrating images over time and normalizing. By combining multiple 2D tracks and scaling the RGB values by depth, the 3D set can be used to generate tracks with colour-scaled depth as shown in Fig. 4 (available: msurj.mcgill.ca/Vol10_1.php).

Quantum Dots

Colloidal Quantum Dots (QDs) are semiconductor nanocrystals whose photoluminescence emission wavelength is proportional to the size of the crystal. (11) Their outer surfaces can be readily conjugated to organic molecules; they are therefore useful as biological labels. (12) Their use in DHMs is, however, not well studied and could suggest a new method for contrast improvement, leading to better tracking abilities.

To test the efficiency of QDs as holographic dyes, we grew multiple batches of *E. coli* together. While we grew one batch under normal conditions as a control, we conjugated another batch with QDs upon reaching half-log growth phase. We then imaged both sample sets and analyzed the images for discernible differences.

Since both samples were cultured from the same source and imaged at equal growth time at equal volume and concentration, they can be assumed to have an equal number of live *E. coli*. Any difference between the observed numbers of bacteria in Fig. 5 can then be associated with the inclusion of QDs.

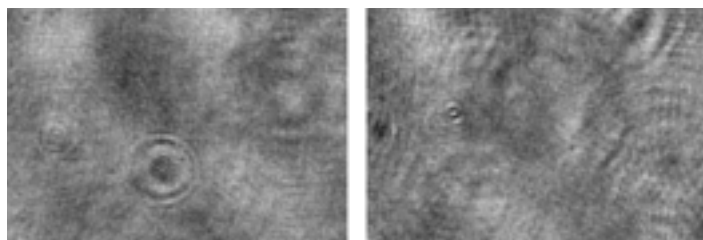


Figure 5. Amplitude reconstructions for *E. coli* sample without (left) and with (right) QD conjugation. Samples were imaged under similar conditions and can be assumed to have equal bug concentration. While individual bugs may be difficult to identify in the images, an overall increase in contrast and variance can be noticed.

To quantitatively measure the change in contrast, we defined contrast ratio as the difference between the maximum and minimum intensity of pixels within a given range. We normalized the dynamic range for all images between 0 and 255, and we analyzed a cross section along y (at fixed x) for contrast ratio. This let us plot contrast ratio (against x coordinate as location) for each set of image as shown in Fig. 6 (image available: msurj.mcgill.ca/Vol10_1.php).

By averaging the measurements over x and t , we calculated average contrast ratio to be $131 (\pm 3)$ for the control sample and $143 (\pm 5)$ for the QD sample, thus leading to a 9% increase in contrast ratio. Repeating the measurements with x and y interchanged yielded similar results: 128 for the control sample and 145 for the QD sample, resulting in a 13% increase. Therefore, it can be seen that there is a small but definite increase in contrast with the use of Quantum Dots.

Conclusion

The dual-beam, off-axis holographic microscopy can be used to image

bacteria in the micron range. The MZ interferometer instrument has a 380 x 380 x 600 (μm) three-dimensional field of view and can maintain nominal < 0.8 mm resolution within this volume. However, the contrast between bug and background is not optimal for tracking and leads to a false positive rate of around 86% currently.

Conjugation of the bugs with Quantum Dots increases the contrast ratio as defined by 11.26 (± 2.06) %. While this change is not large, it does suggest that use of Quantum Dots can increase the contrast for Digital Holographic Microscopes. In further study, we hope to replace the bacteria with micron-size beads and image the samples with and without Quantum Dot conjugation. Unlike live bacteria, the beads will have a fixed concentration which can be found to a high precision, and can be used to quantify change in contrast with QD ratio.

In an additional venue towards improvement, the hardware team behind the microscope is attempting to reduce aberrations present in the current system to improve contrast, which should allow for better imaging and tracking results. A more compact system, named the Common Mode, is being designed. This system includes collimated optical paths instead of an MZ interferometer. Assuming that these changes improve imaging capabilities and allow for successful tracking, the microscope will be applicable in various fields and can be used remotely for studies of microbial organisms.

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Research Article

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Mutation of the Glc-2 Gene May Confer Dominant Ivermectin Resistance

Keywords

Ivermectin: a broad-spectrum anti-parasitic drug frequently prescribed to treat worm infestations

Drug resistance: a phenomenon wherein selective pressure applied by a drug filters susceptible pathogens and leaves resistant pathogen unharmed; leads to reduced drug efficacy

glc-2: a key gene in parasitic worms that encodes an alternate subunit for a membrane channel; reduces channel recognition by ivermectin

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Abstract

Background: Ivermectin is a widely used anti-parasitic drug that binds to and activates glutamate-gated chloride channels (GluCl_s), giving it its nematocidal (nematode-killing) properties. Due to excessive use of ivermectin, frequent cases of resistance to this nematicide are being reported, suggesting that ivermectin is beginning to lose its efficacy. This project seeks to study whether a mutation of the *glc-2* gene, which encodes for a β subunit of the GluCl channel, confers ivermectin resistance. We hypothesize that a *glc-2* mutation achieves nematicide resistance by creating a defective GluCl channel that cannot bind to ivermectin.

Methods: We used classical genetics to obtain the desired mutants from stock worms. We then tested the worms for resistance profile using ivermectin sensitivity assays. Finally, we examined *in vivo* interactions by expressing relevant RNA in a heterologous system and performed electrophysiological recordings.

Results: We were able to demonstrate that presence of the defective *glc-2* leads to increased resistance profiles when given the chance to associate with select GluCl α subunits (e.g. AVR-15). We also demonstrated that co-injection of *glc-2* and *glc-3* compromises GluCl response to L-glutamate, a critical indicator of channel functionality.

Conclusion: Our results lend strong support to our hypothesis that *glc-2* is able to interact with certain α subunits of GluCl to confer ivermectin resistance. This finding provides a framework for future dominant ivermectin resistance studies.

Introduction

Onchocerciasis is a disease found almost entirely in Sub-Saharan Africa, where it poses significant health concerns, with an estimated incidence of at least 25 million infections and 123 million individuals potentially at risk. (1) Ivermectin was developed as a broad-spectrum antiparasitic for veterinary use that has proven to be extremely effective in the treatment of this disease in humans, and was subsequently marketed for human use under the trade name Mectizan. Since its establishment in 1987 by Merck & Co., the Mectizan Donation Programme has achieved impressive gains in onchocerciasis control. (2) Ivermectin has particularly attractive pharmacology as it targets glutamate-gated chloride channels (GluCl_s), which are invertebrate-specific and pose minimal repercussion to the mammalian host. (3) Ivermectin is undoubtedly one of the largest public health success stories of the 20th century, and today has widespread applications in the control of other nematodiasis (e.g., elephantiasis) and ectoparasites (e.g., scabies); it remains a mainstay for parasite control in veterinary medicine and is currently on the List of Essential Medicines put forth by the World Health Organization (WHO). (4-6)

Unsurprisingly, due to ivermectin's appealing pharmacological properties and extensive applicability, the drug has been very intensively used. This has led to the emergence of resistance in many animal hosts, and most recently, in humans. (7-10) The continued effectiveness of ivermectin relies critically upon our ability to keep resistance under control. Our lab has been interested in the emergence of such resistance mechanisms, and using *C. elegans* as a predictive model, we were able to identify critical GluCl α (GluCl α) subunit genes (*avr-14*, *avr-15*, *glc-1*, and *glc-3*) which, if mutated, confer recessive resistance to ivermectin in a synthetic manner.

(11) Nonetheless, these results fail to account for cases in which the resistance appears dominant. (12) We recently isolated via mutagenesis *vu16*, a point mutation allele in the *glc-2* gene that encodes for a β subunit of the GluCl channel. This mutation has proven to be particularly interesting, as it confers synthetic hyper-resistance to ivermectin in a semi-dominant fashion in a 50 μ g/mL ivermectin screen (unpublished data). Mutations in *glc-2* may provide us with a better understanding of how dominant ivermectin resistance could emerge in an invertebrate model.

As GluCl α subunits seem to play a role of great importance in ivermectin binding (all relevant GluCl mutations identified thus far encode for α subunits, as mentioned previously), we hypothesize that the *vu16* point mutation achieves resistance by creating a defective GLC-2 that associates with select α subunits constituting the GluCl, thereby "poisoning" channel function. This poisoning mechanism has been similarly described to account for resistance to levamisole, thus providing a strong precedent for our hypothesis. (13) We validated our hypothesis using a combination of genetics, drug assays and electrophysiology.

Methods

To avoid confusion, italicized terms denote the gene(s) mutated in a given strain (if wild-type forms were used, they will be followed with 'wt'); capitalized words, with the exception of GLC-2 (which is the defective subunit encoded by *glc-2*), denote a functional subunit in its wild-type form. Unless otherwise indicated, the specific mutant alleles of the genes of interest were:

Found on Chromosome I	Found on Chromosome V
<i>glc-2</i> (<i>vu16</i>), <i>avr-14</i> (<i>vu47</i>), <i>dpy-5</i> (<i>e61</i>), <i>unc-57</i>	<i>avr-15</i> (<i>vu227</i>), <i>glc-1</i> (<i>pk54::Tc1</i>), <i>glc-3</i> (<i>ok212</i>), <i>unc-76</i> (<i>e911</i>)

Table 1. Mutant alleles used in the course of this research project, either directly affiliated with the experiments or are used as genotyping markers.

Creating *vu16* quadruple mutants using classical genetics

To test for potential associations of GLC-2, we used genetic crosses to introduce *glc-2* into a triple mutant background, with three of the known GluCl α -encoding genes mutated and one retained in its wild-type form. All of the triple mutants were available from previous experimental crosses, therefore a total of four new strains were made.

MUTANTS TO CONSTRUCT	STRAINS USED AS CONTROL
<i>glc-2</i> (<i>vu16</i>) <i>avr-14</i> (<i>vu47</i>) I; <i>avr-15</i> (<i>vu227</i>) <i>glc-1</i> (<i>pk54</i>) V	<i>avr-14</i> (<i>vu47</i>) I; <i>avr-15</i> (<i>vu227</i>) <i>glc-1</i> (<i>pk54</i>) V
<i>glc-2</i> (<i>vu16</i>) <i>avr-14</i> (<i>vu47</i>) I; <i>glc-3</i> (<i>ok212</i>) <i>avr-15</i> (<i>vu227</i>) V	<i>avr-14</i> (<i>vu47</i>) I; <i>glc-3</i> (<i>ok212</i>) <i>avr-15</i> (<i>vu227</i>) V
<i>glc-2</i> (<i>vu16</i>) <i>avr-14</i> (<i>vu47</i>) I; <i>glc-2</i> (<i>ok212</i>) <i>glc-1</i> (<i>pk54</i>) V	<i>avr-14</i> (<i>vu47</i>) I; <i>glc-2</i> (<i>ok212</i>) <i>glc-1</i> (<i>pk54</i>) V
<i>glc-2</i> (<i>vu16</i>); <i>glc-3</i> (<i>ok212</i>) <i>avr-15</i> (<i>vu227</i>) <i>glc-1</i> (<i>pk54</i>) V	<i>glc-3</i> (<i>ok212</i>) <i>avr-15</i> (<i>vu227</i>) <i>glc-1</i> (<i>pk54</i>) V

Table 2. *glc-2* is brought into backgrounds with classical glutamate-gated chloride channel subunit mutations. A total of 8 strains were used in the ivermectin sensitivity assays, including the 4 *vu16*-containing quadruple mutants and their corresponding wild-type *glc-2* controls, in order to test for potential GLC-2 associations with each individual GluCl α subunit implicated in IVM resistance.

Of available strains, we chose the D621 mutant strain (*glc-2* *avr-14* I; *him-8*(*e1489*) IV; *avr-15* *glc-1* V), which was heterozygous for *him-8*. Progeny from 15 worms were used to eliminate *him-8*.

To obtain *glc-2* *avr-14* I; *glc-3* *avr-15* V, we first crossed N2 males to JD3 (*dpy-5* I, *unc-76* V), heterozygous male F1s to JD624 (*avr-14* I; *avr-15* *glc-3* V), then F2 male progeny to *glc-2* *avr-14* I; *unc-76* V. We constructed this strain in parallel by crossing JD621 to JD3, singling wild-type looking progeny, and eliminating *him-8*. Next, we singled out *unc* F2s to ensure that no F3 *dpy* progeny could be found. From this cross, we singled wild-type looking worms that threw F4 *dpy*. From these *dpy*-containing plates, we singled 15 wild-type looking F4s, and eliminated *unc*, *dpy* and selected candidates on 20 ng/mL ivermectin. We confirmed mutations via either gel electrophoresis (*glc-3*) or Sanger sequencing (*glc-2*, *avr-14*, *avr-15*).

To obtain *glc-2* *avr-14* I; *glc-3* *glc-1* V, we heat shocked our stock strain JD366 (*avr-14* I; *glc-3* *glc-1* V) as described to generate males. (20) We crossed these to JD3, and selected male F1 progeny and crossed them to *glc-2* *avr-14* I; *unc-76* V. We then singled wild-type looking F2 worms that threw *dpy*, then wild-type looking F3s from these plates. We then eliminated *unc*, *dpy* worms. We confirmed mutant alleles by PCR followed by either gel electrophoresis or sequencing.

The first step in obtaining *glc-2* I; *glc-3* *avr-15* *glc-1* V was to generate a strain with only *glc-2* on the first chromosome. To do so, we crossed N2 males to *unc-57*, crossed F1 heterozygous males to JD429 (*glc-2* *dpy-5* *avr-14* (*ad1302*) I; *avr-15*(*ad1051*) *glc-1* V), and singled wild-type looking F2s that threw both *unc* and *dpy* progeny. The F2 singled worms were allowed to self-fertilize and we isolated 200 F3 progeny to locate an absence of either *unc* or *dpy* phenotypes in the F4 population, which is indicative of recombination (occurs at roughly 2% frequency).

The next step involved eliminating all the undesired mutations from chromosome V. First, we crossed N2 males to JD3. Then we crossed F1 males to the *glc-2* mutant. We singled wild-type looking F2s that threw both *unc* and *dpy*, and eliminated *unc*, *dpy* via propagation. We brought the *glc-2*

background into JD300 (*glc-3* *avr-15* *glc-1* V) to get the desired strain, and confirmed all mutations via PCR followed by sequencing or gel electrophoresis.

Ivermectin Sensitivity Assays

We performed ivermectin sensitivity assays as described by Dent et al. with slight modifications. (14) We prepared eggs using the standard alkaline method and resuspended in M9 buffer (42mM Na₂HPO₄, 22mM KH₂PO₄, 86mM NaCl to 1L dH₂O), and 50-100 eggs plated to each NGM plate. Each triple mutant was compared to its quadruple mutant counterpart with the extra *vu16* mutation introduced. We determined the percentage of gravid adults after 4 days of growth at ambient temperature (~25°C) with the individual agar plates divided into 9 sectors to facilitate counting. Concentrations of ivermectin were 0.01ng/mL to 50µg/mL. We constructed concentration-response curves using Igor Pro software (Wavemetrics, Lake Oswego, OR, USA) and fitted to the Hill Equation provided in their function set:

$$\text{base} + (\text{max} - \text{base}) \div [1 + (\text{xhalf} \div \text{x})^{\text{rate}}]$$

To ensure consistency, all agar plates were uniformly seeded with ~0.5mL of HB101 bacteria. Experimental and control strains for each set of mutants were L1 synchronized and counted simultaneously.

RNA Expression Followed by Two-Electrode Voltage Clamp (TEVC)

We selected *Xenopus laevis* females without past surgical history from stock and extracted oocytes under anaesthesia (0.15% MS-222, Sigma, Oakville, ON). The oocytes were subsequently immersed in 2mg/mL collagenase (Sigma) in OR2 buffer (82mM NaCl, 2mM KCl, 1mM MgCl₂, 5mM Hepes to pH 7.5) for 2hr at ~25°C with shaking to remove the follicular layers and facilitate injection. We transferred partially de-folliculated oocytes to a 10cm agar plate containing ND96 (96mM NaCl, 2mM KCl, 1mM MgCl₂, 1.8mM CaCl₂, 5mM Hepes, pH 7.5) and replaced the buffer 3 times to ensure removal of any residual follicular layer.

We extracted RNA using the mMessage mMachine T7 kit (Life Technologies, Carlsbad, CA, USA). Next, we injected each oocyte with 500ng of cRNA (250ng if two cRNA types were simultaneously injected) using a Drummond Nanoject microinjector (Broomhall, PA, USA) equipped with 90mm flared microinjection needles (Harvard Apparatus Canada, Saint-Laurent, QC). We injected fifteen to twenty eggs per experiment to ensure there were enough expressing oocytes for data acquisition. Table 3 depicts the GluCl cRNA that were injected, along with a short description for the rationale.

Injected RNA	Rationale
<i>glc-2</i> (wt)	Control, it was already demonstrated that the wild-type <i>glc-2</i> can form homomeric channels in oocytes*
<i>glc-3</i> (wt)	Control, it was already demonstrated that <i>glc-3</i> can form homomeric channels
<i>glc-2</i> <i>vu16</i>	Control, it was already demonstrated that <i>glc-2</i> <i>vu16</i> is unable to form its own channel (unpublished)
<i>glc-2</i> (wt) + <i>glc-3</i> (wt)	Control, want to see whether co-injection of both cRNA leads to expression of heteromeric channels
<i>glc-2vu16</i> + <i>glc-3</i>	Experiment, if hypothesis true, then the channels formed (if at all) would depict compromised functionality

Table 3. Oocyte injections and rationales. A total of 5 oocyte group was each injected with a different set of cRNA (s) and subsequently recorded using electrophysiological techniques. *Source: Cully et al. (1994)

We incubated injected oocytes at 15°C for 2 days before proceeding to recordings, where eggs were re-introduced to fresh ND96 saline and held at a membrane potential of -80mV. Voltages were recorded using a Axoclamp 2B voltage clamp (Axon Instruments, Foster City, CA, USA). Glutamate was dissolved in ND96 and diluted to different concentrations before being sequentially administered to oocytes. Strong responses, defined by a significant current dip (>500nA) upon application of 1mM glutamate, were indicative of functional GluCl channel formation and results were acquired using Clampex 10 Data Acquisition Module (Axon).

Results

Differential ivermectin resistance across the four different quadruple mutants

The control strains *avr-14* (I); *glc-3 avr-15* (V), *avr-14* (I); *glc-3 glc-1* (V), *avr-14* (I); *avr-15 glc-1* (V) and *glc-3 avr-15 glc-1* (V) exhibited ivermectin EC₅₀s of 123.23 ± 9.23 , 2.43 ± 1.07 , 500.31 ± 26 , and 5.87 ± 0.17 ng/mL respectively, results that are comparable to and consistent with past ivermectin sensitivity trials (unpublished findings). We observed a modest increase in resistance when we introduced *glc-2* into *avr-14* (I); *glc-3 avr-15* (V), as denoted by a slight rightward shift of the concentration-response curve. In contrast, we observed hyper-resistance to ivermectin when *glc-2* was introduced into either the *avr-14* (I); *avr-15 glc-1* (V) or *avr-14* (I), *glc-3 glc-1* background. Finally, *glc-2* (I), *glc-3 avr-15 glc-1* (V) and *glc-3 avr-15 glc-1* (V) showed the same level of low synthetic resistance to ivermectin until a slight difference at maximal IVM concentration, a phenomenon that we failed to explain. The EC₅₀s of *glc-2 avr-14* (I); *glc-3 avr-15* (V), *glc-2 glc-2 avr-14* (I); *avr-15 glc-1* (V) and *glc-2 avr-14* (I); *glc-3 glc-1* (V) were indeterminable within our assessment limits, as they showed robust resistance and never dropped to baseline level, even at 50 µg/mL ivermectin. However as a proof-of-principle, we believe that these initial data are sufficient. These results are summarized in Fig. 1.

Co-injection of *vu16* with *glc-3* compromises response to glutamate

To validate our findings further, we elucidated subunit interactions by co-injecting GluCl cRNAs into *Xenopus* oocytes. Since GluCl activation is mediated by glutamate, a robust response after its application is a reliable indicator of channel functionality. As a control, we administered glutamate to three un-injected oocytes, none of which responded (data not shown). GLC-2 GluClβ homomers responded to concentrations of glutamate as low as 50 µM. GLC-3 homomers were slightly less sensitive, with responses recorded at 100 µM glutamate. The heteromeric channel formed by GLC-3 and GLC-2 was significantly more sensitive to glutamate, with responses evident at 10 µM. Glutamate concentrations ranged from 2 µM–2 mM and their effects in oocytes were concentration-dependent, consistent with past observations made in our lab with glutamate application to oocytes expressing *Haemonchus contortus* (Hc) GluClas (15). Oocyte responses with the least noise interference for each group are shown in Fig. 2. Each recording was repeated three times in three separate oocytes.

None of the 10 oocytes injected with GLC-2*vu16* cRNA responded to glutamate (data not shown). More interestingly, however, *glc-2* co-injection with *glc-3* wt dramatically compromised the glutamate response, to the point of nearly abolishing it. The highest glutamate concentration available (5 mM) was unable to induce a current >200 nA, a marginal response compared to the much larger currents (>1000 nA despite much lower glutamate administration) recorded in the other experimental oocyte groups.

Discussion

GLC-2 potentially interacts with GLC-1, AVR-15 and GLC-3, but not AVR-14

We separately mutated three GluClα subunits while maintaining one functional subunit. *glc-2 avr-14* (I); *glc-3 avr-15* (V), *glc-2 avr-14* (I); *glc-3 glc-1* (V) and *glc-2 avr-14* (I); *avr-15 glc-1* (V) worms all exhibited greater resistance to ivermectin upon substitution of *glc-2* wild-type background with *glc-2*. This result suggests that GLC-2*vu16* likely interacts with wild-type GLC-1, AVR-15, and GLC-3, respectively, and that increase in ivermectin resistance resulted from disruption of GluCl channel integrity upon heteromeric association with the defective GLC-2*vu16* protein. Since the presence of GLC-2*vu16* does not seem to affect ivermectin resistance of the strain *glc-3 avr-15 glc-1* (V), then by the same reasoning GLC-2*vu16* and wild-type AVR-14 subunits are unable to associate with each other. Thus, from our initial concentration-response results, we propose that GLC-2*vu16* can form heteromeric channels with GluClα subunits, and that a mechanism exists that determines which subunits it can

interact with.

Another interesting observation is that the level of synthetic ivermectin resistance conferred is variable, depending on which subunits are available for interaction (e.g., moderate increase for GLC-1 and substantial increase for AVR-15/GLC-3). While the exact mechanism is poorly understood, we believe that it may be attributable in part to the localization of the different α subunits. Through past mapping and laser ablation studies, we were able to construct a hypothetical model that proposes different pathways to attain ivermectin sensitivity. (12) A slightly modified depiction of the model is shown in Fig. 3. Since AVR-15 subunit-containing channels are localized in the pharynx, it plays a much greater role mediating essential physiological functions, especially feeding. Meanwhile, AVR-14 and GLC-1 are localized in the extrapharyngeal neurons, which play somewhat of a supplementary role, making it more difficult to develop hyper-resistance upon interaction with GLC-2*vu16*. It is also noteworthy that *glc-1* is *C. elegans* specific, and as such may have evolved new functionality in other tissues, thereby reducing its ability to interact with GLC-2. The exact expression profile of GLC-3 is debated and not shown in the model, but evidence suggests that it is localized in the olfactory AIY interneurons, which may play an important role in olfaction-regulated turning behaviour (16).

GLC-2 interacts with and poisons GLC-3 in a heterologous system

Our concentration-response data can only provide a preliminary assessment of ivermectin resistance upon *glc-2* incorporation into a mutant background strain. Ion channel expression is necessary to provide further insights into possible *in vivo* subunit interactions. Activity of *glc-2* wt/*glc-3* wt co-injection is different from their individual expressions, thus demonstrating that GLC-2, in its wild-type form, does form a heteromeric channel with GLC-3. As such, the lack of significant channel activity when *glc-2* was co-injected with *glc-3* wt was particularly noteworthy. These co-injection results have three possible interpretations: [1] GLC-2*vu16* cannot assemble with GLC-3. While this is a plausible scenario, it does not explain our data as GLC-3 should still be expressed normally, and we would expect recordings consistent with a GLC-3 homomeric channel; [2] GLC-2*vu16* is able to form functional heteromers with GLC-3. This is very unlikely, since that would again lead to robust current recordings with glutamate administration; [3] GLC-2 is able to associate with GLC-3, but creates a heteromeric channel with compromised functionality. Our data strongly support the last possibility, a result that is consistent with our poisoning hypothesis. Glutamate activation was possible with GLC-3 subunits alone, but this ability was severely compromised when a defective GLC-2 was introduced.

Limitations

Time was the most significant limitation for our project. Obtaining the quadruple mutants was particularly challenging and time-consuming. Additionally, the *glc-2* (without *avr-14*) background on chromosome I was extremely difficult to acquire due to low recombination rates, and there were technical issues screening for the *glc-1* mutant allele using PCR. The ivermectin sensitivity assays and electrophysiological recordings had internal replicates, but were performed once. Validation of our hypothesis would require additional technical replicates. The oocyte recordings investigated channel expression with only GLC-3 and GLC-2; co-injection with *avr-15* wt, *avr-14* wt and *glc-1* wt has not been adequately examined.

We have shown a “poisoning” effect of GLC-2*vu16* on heteromeric channels with GLC-3 in *Xenopus* oocyte electrophysiology. If this occurs *in vivo*, it can explain the partially dominant hyper-resistant phenotype observed. We have yet to demonstrate that this is in fact the case, but can state that *glc-2* is a potential key player in ivermectin pharmacology. With GLC-3 in *Xenopus* oocyte electrophysiology. If this occurs *in vivo*, it can explain the partially dominant hyper-resistant phenotype observed. We have yet to demonstrate that this is in fact the case, but can state that *glc-2* is a potential key player in ivermectin pharmacology.

Future Directions

Our goals will seek to address the current limitations with our dataset. One priority is to expand our recording experiments to include cRNA co-injections of *glc-2* with *avr-15* wt, *avr-14* wt and *glc-1* wt. Our hypothesis would be supported if we can show that AVR-14 expression is unaffected by the presence of GLC-2, while all other co-injection groups exhibit a much reduced or non-existent glutamate response.

Our second goal is to generate four additional quadruple mutants of *glc-2* using the mutation ok1047, which is a null whole gene deletion rather than a single point mutation. (18) This means we can study mutants that do not produce GLC-2, and we will screen these strains in parallel with the *glc-2* vu16 quadruple mutants and the *glc-2* wt GluCl α triple mutants. For our poisoning hypothesis to be supported, we would expect to see that the *glc-2* deletion mutants are as sensitive to ivermectin as the triple mutants, and do not exhibit the hyper-resistance associated with *glc-2* vu16.

Conclusion

Ivermectin remains a critically important drug for the treatment of parasitic diseases. With the current focus shift of African Programme for Onchocerciasis Control (APOC) from containment to elimination, and plans to increase ivermectin mass administration to the public, understanding the mechanisms of ivermectin resistance is paramount. (19) Not only will it help provide insight on current drug administration practices, but it will also aid in the development of new compounds that can bypass the structural GluCl mutations (e.g., using medicinal chemistry to optimize access to truncated target sites). Our preliminary results with *glc-2* have helped shed new light on this area, and set the foundation for future research in dominant ivermectin resistance, as well as mechanisms of parasite resistance in general. With ongoing research in this direction, it is our hope that ivermectin can continue being a therapeutic success as it has been forty years past, forty years into the future.

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Figures

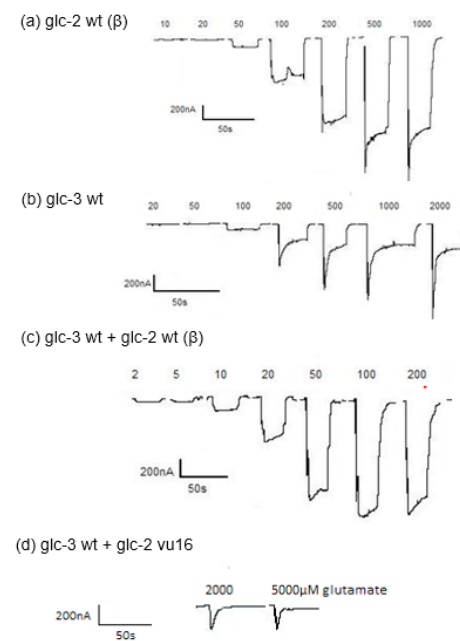


Figure 2. Glc-2 association compromises response to glutamate when co-injected with *glc-3*. Electrophysiological recordings (TEVC) from RNA-injected oocytes and tested for L-glutamate response at progressively increasing concentrations. Oocytes injected with *glc-2* wt (a), *glc-3* wt (b) or co-injected with *glc-3* wt and *glc-2* wt (c) exhibited much greater L-glutamate sensitivity than those co-injected with *glc-3* wt and *glc-2* vu16 (d).

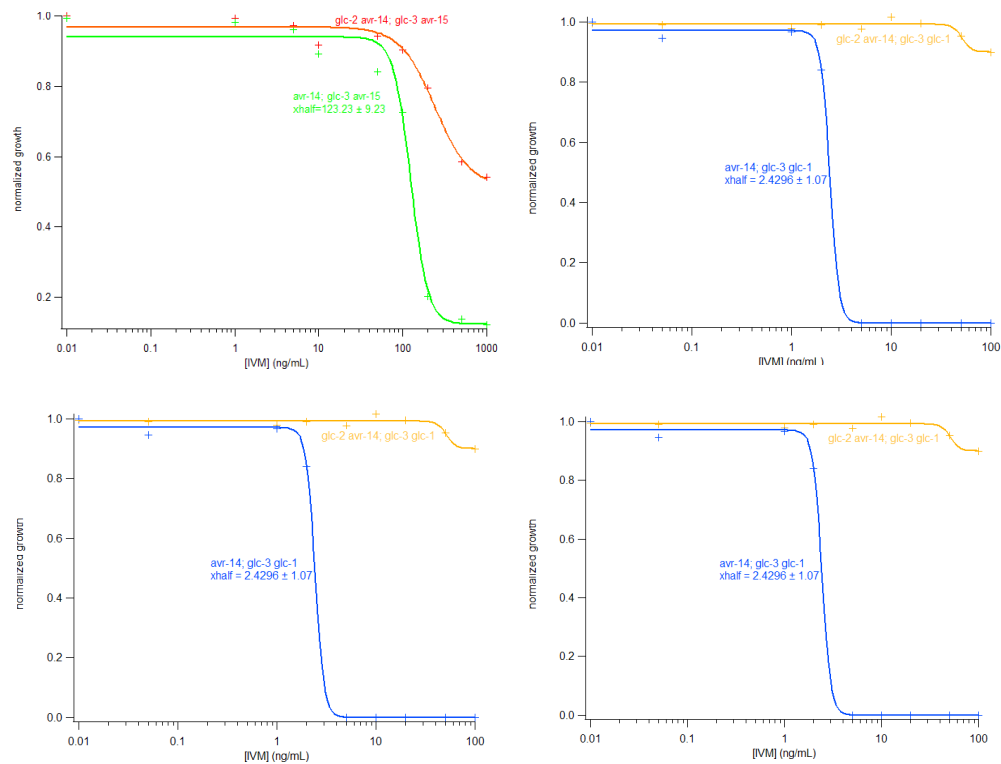


Figure 1. A defective GLC-2 interacts and poisons select subunits that constitute the GluCl channel. Our results reveal that a defective GLC-2 leads to a modest increase in resistance with a functional GLC-1 (a), a substantial increase with a functional AVR-15/GLC-3 (b and c), and no change at all with a functional AVR-14 (d).

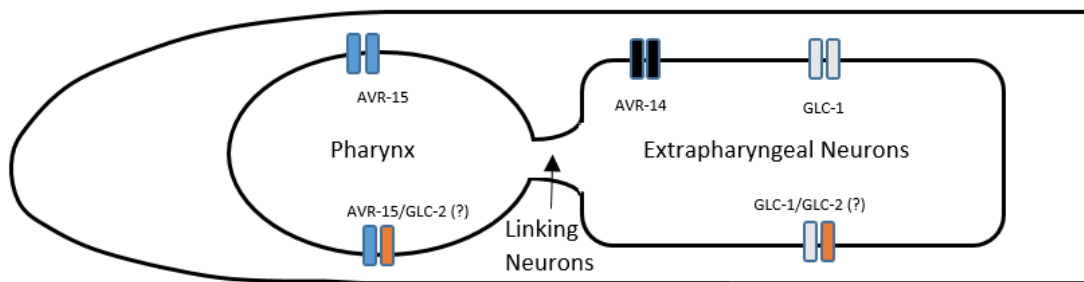


Figure 3. Postulated sites of interaction of GLC-2 in the ivermectin pathway AVR-15 subunits are expressed in pharyngeal GluCl channels while AVR-14 and GLC-1 subunits are expressed in extrapharyngeal neurons. The exact expression profile of GLC-3 is currently debated and thus not shown.

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Keywords

Fagus grandifolia: a species of
beech trees native to northeast-
ern North America, important to
forestry

Beech bark disease: an infectious
disease afflicting beech trees,
caused by fungal infection at in-
sectal wounds

Mont St-Hilaire: a mountain lo-
cated in Southern Québec, home
to Gulf of St. Lawrence lowland
forests

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Tree Diversity has Limited Effects on Beech Bark Disease Incidence in American Beech Population of Mont St-Hilaire

Abstract

Background: American beech trees (*Fagus grandifolia*) exist in many areas in northeastern North America. Beech bark disease (BBD) is caused by a scale insect and bark-killing fungus (*Cryptococcus fagisuga* and *Nectria* spp.). We aim to study the correlation between diversity and the presence of BBD, and predict that tree diversity in Gault's Nature Reserve in Mont St-Hilaire (MSH), Québec decreases the presence of BBD and that *F. grandifolia* density would increase the presence of this disease.

Methods: We randomly chose 15 sites for sampling of individual tree species. *F. grandifolia* trees were identified as "healthy" or "infected". Simple regressions, ANOVA, two and three-way interaction, linear mix effect model, and paired t-test were performed using R and Excel.

Results: Our results show no significant correlation of infected individuals and total number of either *A. saccharum* or *A. pensylvanica*, unless analyzed with a linear mixed effect model ($p=0.0256$). However, there was a strong, positive correlation between the number of infected trees and the density of *F. grandifolia* ($R^2=0.6712$), and this relationship was stronger in disturbed areas compared to undisturbed areas in the reserve ($t=2.0492$, $p=0.047$, $t_{critical}=2.0211$).

Conclusion: We found beech tree density and habitat disturbance, but not community diversity, to have a significant positive effect on Beech Bark Disease infection rates.

Introduction

The American beech (*Fagus grandifolia*) is a shade tolerant, long-lived canopy tree species that is the only species of the genus known to exist in North America. (1-3) The American beech is considered to be a foundation species that influences the ecosystems in which it grows by providing different resources such as understory shade, leaf litter, and food for the wildlife. (1-6) Thus, it is an ecologically important species that coexists with other dominant canopy species such as Sugar maple (*Acer saccharum*) in Northern hardwood, mixed deciduous, and temperate forests in northeastern North America. (2, 3, 6, 7)

The American beech tree is a monoecious species that can reproduce clonally by root sprouts under situations of stress. (1, 8) After a disturbance resulting from tree injury or disease, this vegetative reproduction leads to dense thickets of clonal beech sprouts. (9, 10) This phenomenon makes clonal beech thickets an important determinant of local biodiversity as sprouts can readily come to dominate a disturbed area. (4)

Towards the end of the 19th century, when nursery stocks of ornamental European beech (*F. sylvatica*) were brought to Halifax, Beech Bark Disease was introduced to North American forests from Europe. (11, 12) Since its appearance in Nova Scotia in 1911, the disease has spread outwards within the American beech's range. (11, 13, 14) Today, the disease can be found throughout northern hardwood forests from the Canadian Maritimes to southern Quebec and Ontario. (15, 16)

Beech Bark Disease is an insect and fungus complex that begins with injury to the tree's bark by an alien scale insect, *Cryptococcus fagisuga*, followed by the more deadly infiltration of bark killing pathogens in the Ascomycetes family of the genus *Neonectria* whose spores are propagated

by wind and water. (11, 17) As larvae, the scale insects pierce the beech's bark to feed on the tree's phloem throughout the late summer. (17) Crawlers develop between June and September, and are easily dispersed by wind, wildlife, and even humans. (6, 11, 18, 19) The insects overwinter until they can molt during the spring into wingless adults. (11, 17, 20) Insect feeding causes tree cells to desiccate locally and becomes clearly visible on the trunk as clusters of small white spots of dried sugary sap. (11) Once the bark has been penetrated, fungal species *Neonectria faginata* and *Neonectria ditissima* infiltrate and cause severe cankering and formation of callus tissues that cause the tree to be girdled and eventually killed. (13, 21) The presence of the scale alone is not fatal to beech trees, though it highly predisposes the bark to infection from *Nectria*. (4, 11)

Of all the Monteregian Hills, Mont Saint-Hilaire (south of Montreal, Quebec) is least disturbed by human activity and the richest in terms of natural history and cultural interest. It is also the location where Beech Bark Disease was first observed in the late 1980s. (22) Its northern hardwood community is an appropriate study area because its forests contain many diverse microhabitats that could potentially foster different levels of tree diversity. The variation in diversity could correlate with the presence of Beech Bark Disease. In the years ahead, forest communities found at Mont Saint-Hilaire are expected to undergo significant changes in species composition, some of which can be attributed to the impacts of Beech Bark Disease. (22)

The objective of this study was to investigate the relationship between forest diversity and the presence of Beech Bark Disease in Mont Saint-Hilaire. Recent studies have linked biodiversity loss with increased rates of disease and parasite severity in both animal and plant systems. (23, 24) With this in mind, we predicted that increased tree diversity in the reserve will correlate negatively with the presence of Beech Bark Disease, because

we expected community diversity to slow down disease spread. (25, 26)

Methods and Materials

Data was gathered from the 26th to 28th of August 2014. We randomly selected 15 sites on the reserve with a random generator (www.random.org) from an alphanumeric gridded map of Mont Saint-Hilaire (Fig. 1). Each site measured 500 m by 500 m. Eight sites fell in disturbed areas, and seven in undisturbed areas. Disturbed areas were defined as areas where the public has access to trails which are wide and frequently visited by the public or the reserve's staff. Undisturbed areas were defined as areas where the public does not have access, with narrow or nonexistent trails.

Each site contained at least three circular 100 m² quadrats. Each quadrat was between 20 m and 40 m away from the closest trail. Using a compass and key landmarks, such as trails and streams, to reach the approximate centre of each site, the "ignorant man" technique was then used to select the three separate sample quadrats. (27) We counted the number of trees of each different species in each quadrat. We considered only mature individuals having a breast height diameter larger than 8 cm, measured with a 25 cm long string. We noted whether each individual beech tree was infested using the presence of white traces of scale insect penetration as a proxy for Beech Bark Disease. We also made notes on subjective observations regarding the disease's severity.

For every statistic test performed, we assumed that trees, quadrats, and sites are independent data points and that there is no overlap between plots and sites (random sampling). All levels of significance were set at $p < 0.05$.

Diversity index and correlation analysis

We used Shannon's and Simpson's indices as measures of diversity as they are direct measures of ecological diversity and account for the community weight and the species abundance. (18, 29) We calculated these indices on Microsoft Excel (2007) for every site.

As many of the data sets were not normally distributed and were therefore analyzed non-parametrically, we used Kendall's tau rank to determine whether the number of infected individuals was correlated with Shannon's index of diversity, Simpson's index, number of American beech, number of sugar maple, or number of striped maple.

T-test

We performed t-tests in R (version 3.1.1 2014) to identify differences in infection frequencies between the categorical split between sites (disturbed and undisturbed).

Results

A total of 49 plots were recorded, accounting for 560 trees sampled. From this data, 39% were American beech (*F. grandifolia*), 38% were Sugar maple (*A. saccharum*), and 7% were Striped maple (*A. pensylvanicum*); the remaining 16% accounts for different species of trees that we did not consider in our calculation as they occurred in very reduced numbers across plots. From the proportion of American beech counted in this experiment, 69% showed signs of *C. fagisuga* invasion via the presence of dried sugary sap and were therefore assumed to be infected with Beech Bark Disease.

Correlation Analysis (Kendall's Tau Hypothesis)

When performing the Kendall's tau calculations where $\tau_{crit}=0.163$ for all cases, we found that diversity indices were not correlated to the presence of infection in any way ($\tau_{Shannon}=0.061$; $\tau_{Simpson}=0.140$). However, the density of number of American beech is correlated to the number of infected trees ($\tau_{beech}=0.752$). Furthermore, the number of species and the number of trees per site are also correlated ($\tau_{\#species}=0.316$; $\tau_{\#trees}=0.434$). The presence of red

maple ($\tau_{r.maple}=0.621$) also showed correlation, and the presence of sugar maple showed no correlation ($\tau_{s.maple}=0.07$)

T-test

When the data were separated categorically by habitat type (disturbed vs undisturbed), a t-test revealed that the mean number of infected beeches in each plot in undisturbed areas (mean = 1.77, sd = 2.14) was significantly lower than the mean number of infected beeches in disturbed areas (mean = 3.67, sd = 4.18, $t = 2.0492$, $df = 40.233$, $p = 0.047$), though the mean number of beeches between the two habitat types did not differ significantly (mean_{disturbed} = 3.636, sd_{disturbed} = 4.20, mean_{undisturbed} = 5.259, sd_{undisturbed} = 3.1403, $t = 1.4224$, $df = 45.085$, $p = 0.1618$). Furthermore, the interaction between beech density and habitat type on number of infected beeches was also significant ($t = -2.516$, $p = 0.0155$) (Fig. 2). This fact directed our attention to a relationship that we did not consider at the beginning of our experiment. The correlation between the number of infected individual by Beech Bark Disease not only increases as the density or number of American beech increases, but has a noticeable and much stronger effect in disturbed areas ($R^2 = 0.7824$) than in undisturbed areas ($R^2 = 0.3158$). This indicated that disturbance could be a major factor driving the prevalence of Beech Bark Disease in Mont Saint-Hilaire (Fig. 3).

Discussions

The objective of this study was to explore the relationship of tree diversity and density on the presence of Beech Bark Disease in Mont Saint-Hilaire. Our analysis indicate that though community diversity has no significant effect on Beech Bark Disease infection rates on Mont Saint-Hilaire, American beech tree density does correlate significantly with disease incidence. In addition to tree density, the impact of habitat disturbance on infection incidence is positive and interacts with density effects on the Beech Bark Disease.

Our data showed no significant correlation between tree diversity and Beech Bark Disease frequency. It is possible that other trees influence the incidence of Beech Bark Disease, for example, by facilitating niches for animals that have been shown to transport the scale insect, but this is beyond the breadth of our study. (28) Although communities of greater Shannon's and Simpson's diversity indices contain greater diversity, it is possible that the areas on Mont Saint-Hilaire we assessed contain habitats that are not different enough from one another to be called different "communities", and that therefore diversity does not play a role. (29) In addition, many past studies that link diversity and infection have measured disease severity rather than disease frequency. Mitchell et al. (2002) also suggested that the effect of diversity on disease might be stronger in natural systems than in their laboratory experiments because differences in diversity would occur at a larger scale. (23) This may well be the case for Beech Bark Disease and Mont Saint-Hilaire's American beech trees. Therefore, an objective and quantifiable measure of disease severity should be recorded in further research.

Although it is difficult to understand the precise community composition effect from the data recovered from this study, the relationship between beech population density and Beech Bark Disease frequency is clear. Though it cannot be said that increased diversity slows down the spread of the infection as initially hypothesized, the number of beech trees in each quadrat, i.e. beech tree density, is highly correlated with the infection density in the quadrats. The trend of increasing disease frequency with increasing beech numbers suggests a density-dependent relationship between Beech Bark Disease and the American beech. Density dependence is a common theme in disease ecology, characterized by increased infection rate with increased host density; that is to say, as more individuals are present in a given area and come into contact with one another, the probability of the disease spreading between individuals increases as well. (30) Density dependence cannot be directly observed by this study, as this would require an analysis of contact rate between infected and non-infected individuals over time. (30) It is therefore possible that our findings are simply a product of the probability of encountering an infected individual

increasing with the total number of individuals encountered. Nevertheless, density dependence is supported by one of the three main factors outlined in early literature regarding the spread of Beech Bark Disease; the density of the stand highly influences the development of the disease. (11) Density dependence is known to affect plant demography from growth to production and mortality. (31) As beech trees lack mobility, the rate at which potential hosts become exposed to the disease (contact rate) is a function of the ability of both the insect and the fungus to contact the tree. The scale's chance of encountering a beech tree increases with the density of beech trees around the point source of infection. (13) Houston (1994) also added that at least 80% of American beech mortality happens where the density of this tree is high, which can support the explanation of density dependency. (13) Other fungal infections similar to Beech Bark Disease have overwhelmingly been found to be density dependent, despite being vector borne, possibly due to the lack of vector (fungal) behavioural variation with increased host density. (2, 31-33)

Alternately, because of the American beech's tendency to produce clonal sprouts via their root system, large, densely-populated patches observed in the field may have been made up of one single individual with many clonal sprouts. (9, 10, 34) This possibility of genetic relatedness was not accounted for in our survey, as each trunk above ground was sampled as a genetically distinct individual. The clonal sprouting capacity of the American beech is likely to influence the overall susceptibility of a so-called "population" of beech trees in an area, as it is known that genetic uniformity in clonal plant populations renders them more susceptible to infection. (35) Past genetic variability in Mont Saint-Hilaire's American beech population has no doubt impacted today's community, considering Beech Bark Disease has been observed on Mont Saint-Hilaire since the 1980s. (22) Nonetheless, selective pressure for disease resistance in trees on the mountain, as well as the root sprouting and as full- or half-sib seedlings cluster due to the species' limited seed dispersal radius may have led to the existence of more or less tolerant individuals and sub-populations on the mountain. This could in turn impact the diversity and composition of the forest. It is for these reasons that genetic analysis of relatedness in sub-populations should be addressed when investigating Beech Bark Disease on Mont Saint-Hilaire. It is necessary to investigate whether the healthy beeches growing around a surviving American beech individual are closely related, or whether the trees growing around a survivor are clones of the same tree, which indicates that a new individual with resistant genes does not exist. This might suggest whether there is inheritance of a resistant phenotype in Mont Saint-Hilaire, and will show whether the spread of disease has already slowed and entered aftermath phase.

Habitat disturbance was also found to significantly correlate with Beech Bark Disease incidence. In addition to density mediated disease transmission, habitat disturbance may facilitate the propagation of Beech Bark Disease. Even though Mont Saint-Hilaire's reserve area is rather small (10 km²), the mountain can be characterized as being highly fragmented, particularly in the disturbed publicly accessible areas. The edge of a habitat is often cited as the area at which abiotic factors, such as wind and temperature changes, act most harshly, and effect which may be amplified if the edge is expanded by fragmentation, a phenomenon that may be especially harmful in small communities such as Mont Saint-Hilaire. (36) The large-scale alteration of the natural forest to accommodate leisure activities on Mont Saint-Hilaire contributes to habitat fragmentation within the forest by breaking up contiguous forested area for trails and roads, generating corridors for Beech Bark Disease to be spread between patches while isolating stands of American beech from dispersal and migration between sub-populations. It is possible that by opening spaces in the forest for pedestrians and cars, the increased wind flow through these corridors could aid the spread of scale insect and fungus, as wind flow speed is known to be a limiting factor to the spread of the disease. (4, 13) Furthermore, *C. fagisuga* crawlers are transported by animals – including humans (11). First insights into Beech Bark Disease spread was the transport of infested logs or firewood by humans. (11, 13, 22) The enormous number of people visiting Mont Saint-Hilaire on a daily basis may serve as vectors transmitting scale larvae and fungal spores around the mountain.

Beyond accelerating the rate of disease transmission, human activity may also increase the severity of the disease. The fungus infects the American beech individuals by penetrating into the compromised cells via the cracks

in the bark. (11) Therefore, it is possible that disturbances by human activities lead to more injuries on the American beech bark, enabling *Nectria* to penetrate the bark tissue and causing more severe Beech Bark Disease.

In further research, it would be important to consider the idea that disturbance and beech density might not be independent factors. It could be possible that density of American beech could increase as a result of disturbance, given its capability to form clonal thickets under stress. (1) Assuming that each susceptible American beech tree has an identical possibility of the scale insect introduction, this result suggests that beech trees within areas containing more disturbances are more severely affected by Beech Bark Disease than those in areas with less human activity. Scale feeding wounds are not a necessary precursor of infection, and the pathogen can even penetrate healthy, previously unwounded tissues, though to a limited extent. (37) Further research should be carried out and take the infection rank of *Nectria* colonies into the consideration. No matter the mechanism, it is clear that disturbance and human interaction with the habitat is related to the propagation of Beech Bark Disease through Mont Saint-Hilaire's forest and has most likely also played a role in its spread throughout North America.

Anthropogenic changes to habitat affecting American beech populations are not limited to direct changes to the physical environment. Climate change due to ever-increasing human consumption of fossil fuel and carbon dioxide emissions and other greenhouse gases will certainly have an impact on the range of both the beech and its parasitic detractors. The successful survival of the beech scale, and thus the disease, from one year to the next is heavily determined by environmental factors. (13) If the environment were to become more hospitable to the insect, it is probable that the disease's range would subsequently expand. (20) Heavy rains that wash larval insects to the ground and cold temperatures inhibit the severity and spread of the disease. (13) During the colder months, as the beech scale overwinters, temperatures of -34°C or lower cause reductions of scale populations found in the following growing seasons. (13, 17) Strong warming has been observed in southern Quebec since 1950; continual warming and mild winters could have disastrous consequences in terms of both disease spread and severity due to failed dieback and higher recruitment rates. (20, 38) This pattern has been observed in other bark pests, such as the mountain pine beetle of British Columbia. (39) Though the scale is limited in its spread northward by range of the American Beech itself, incidents of colonization will likely be more persistent and successful as daily low temperatures increase.

Conclusion

It is important to continue to study the stage of Beech Bark Disease in Mont St-Hilaire, as this research provides powerful insights that could be extrapolated to a larger scale to understand the function of forest communities related to the spread of other diseases, as well as other environment-altering forces. Changes in forest composition and diversity may well play a role in Beech Bark Disease's spread and severity in North America. However, these are but mechanisms of an overlying, human-driven issue. Limited, short-term solutions such as pesticide application are unlikely to provide respite from the advancing front that is Beech Bark Disease. As we advance to the future, human activity will dictate the spread of Beech Bark Disease through North America in the same way human activity initially introduced it to the continent. Unfortunately, the solution will prove to be far more complex than the problem's initial nautical delivery.

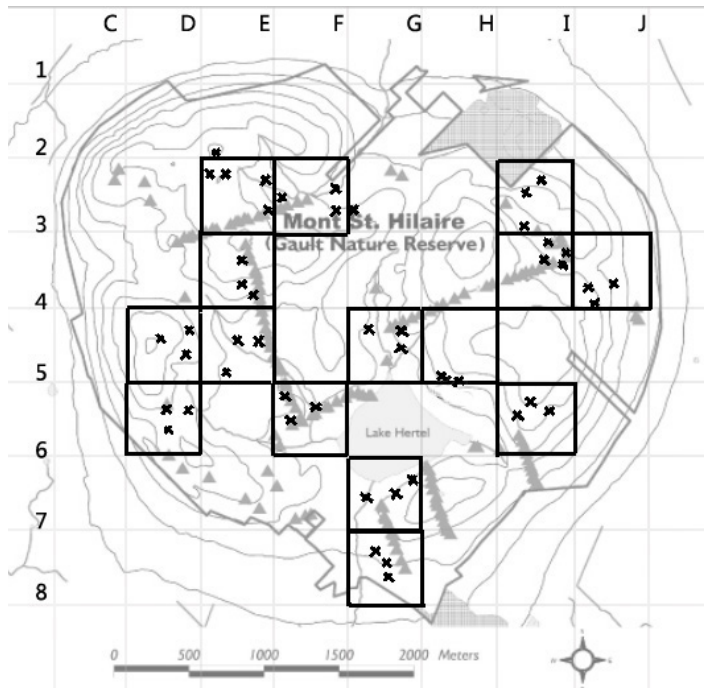


Figure 1. Map of Mont Saint-Hilaire. Sample sites are represented by darkened squares and approximate quadrat locations are represented by 'x's. Figure modified from Arii et al. (2005).

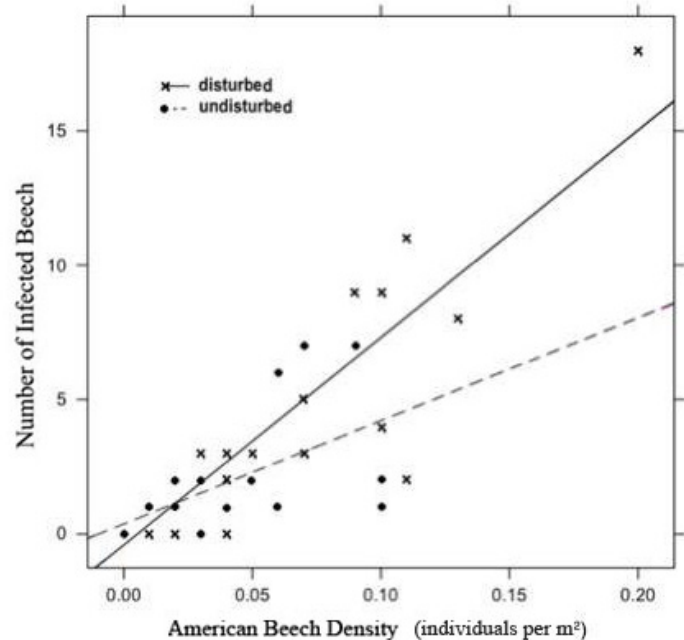


Figure 3. Figure shows the interaction between the number of infected individuals by BBD in disturbed and undisturbed areas with respect to the number of *F. grandifolia*. In disturbed areas, $R^2 = 0.7824$, and in undisturbed areas, $R^2 = 0.3158$.

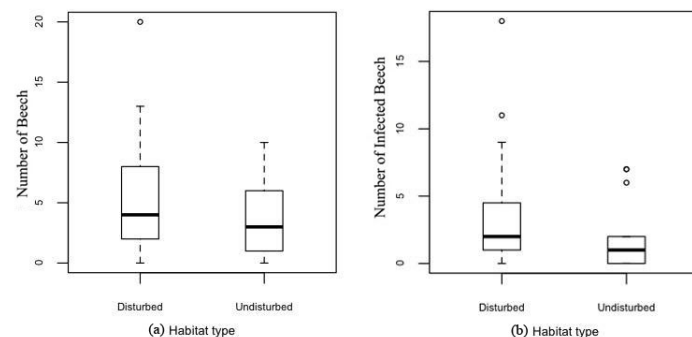


Figure 2. Figure shows the results of T-test performed on the correlation of disturbed and undisturbed habitats on (a) number of American beech; (b) number of infected American beech.

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Keywords

Disorganized attachment: caused by abusive treatment of the child by the part of the primary caregiver; child displays fear and disorientation in the context of separation and stress

DRD4: A G protein-coupled receptor activated by the neurotransmitter dopamine; associated with psychiatric and neurological disorders

Low birthweight: defined as less than 2500 g in newborns

Endophenotype: a concept in psychiatry used to describe hereditary traits associated but not delineated by a genotype

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Preschool Children Without 7-repeat DRD4 Gene More Likely to Develop Disorganized Attachment Style

Abstract

Background: A disorganized attachment style is characterized by fearful and disoriented behaviours when a child is separated from their caregiver. Its presence is an early predictor of the later development of psychopathology, including depression. This study seeks to determine the effects of one environmental factor – the incidence of low birth weight – and one genetic factor – the presence of the long (7-repeat) polymorphism of the dopamine D4 receptor (DRD4) gene – on the development of disorganized attachment between the child and the mother.

Methods: We sampled 251 mother-child dyads from the Maternal Adversity, Vulnerability and Neurodevelopment (MAVAN) project. We measured birth weight and DRD4 genotype, and assessed attachment style using modified separation-reunion procedure.

Results: Birth weight did not have a significant effect on disorganized attachment ($b=-0.001$, $t(230)=0.006$, $p=0.910$). Presence of DRD4 7-repeat polymorphism had a significant negative effect on presence of disorganized attachment style ($b=-1.196$, $t(230)=0.411$, $p=0.004$).

Conclusion: Children without the DRD4 7-repeat allele were more likely to display a disorganized attachment style compared to children with the DRD4 7-repeat allele. This indicates that the 7-repeat allele of the DRD4 gene may serve as a protective factor against disorganized attachment.

Introduction

Overview - Attachment Theory and Psychopathology

It has long been known that the interaction between a child and his or her caregiver has a significant effect on later behavioural and psychological development. (1) In numerous studies, it has been shown that the nature of the early nurturance between caregiver and child has a significantly predictive relationship with outcomes such as infant physiological responsiveness to stressors as well as later cognitive and social development. (2-5)

The original conceptualization of attachment described an evolutionary-based, innate predisposition of the child to seek proximity to and contact with a specific caregiver, most notably when the child is frightened, tired, or ill. (7, 8) Attachment is a dyadic process whereby both the parent and the offspring provide cues and behaviours which strengthen the bond. Ainsworth's research indicated that children may be classified as having one of three primary attachment styles: secure, insecure-avoidant and insecure-resistant. (8) A fourth attachment style has emerged and has been added to this typology: disorganized attachment. (9) Children who are classified as exhibiting disorganized attachment tend to demonstrate fearful and disoriented behaviours in the context of separation and stress. This behaviour is thought to reflect the inability of these children to re-

solve anxiety. (9)

Disorganized attachment is an early predictor of the development of psychopathology in childhood and adolescence. (10) It is associated with externalizing behaviours such as aggression and anti-social personality types, and internalizing disorders such as depression and anxiety. (11) It has been hypothesized that children with disorganized attachment may develop particular schemas that can lead to depression, such as being overwhelmed by particular difficulties or viewing oneself as incapable when facing challenges. (12) Accordingly, recent research finds disorganized attachment to be a potential endophenotype for internalizing disorders such as adult onset depression. (13)

Environmental Factor of Interest – Birth Weight

There has been a significant amount of research conducted on the effects of fetal and infant growth on developmental outcomes. Most of this research focuses on the survival outcomes of 'at risk' infants born with lower birth weights, though research also has looked at effects of very low birth weight (VLBW) on motor, cognitive, behavioural, and emotional development. (14) Risks for later neurological impairments or behavioural problems are heightened and are widely discussed for such infants. (15-22) As well, significant differences in the behavioural and emotional self-regula-

tion of these 'at risk' infants have been reported, with findings of decreased attention, reduced positive affect, and prolonged reaction time to stimuli. (23-27)

Birth weight has been considered to reflect pressures on personal growth and stress exposure during pregnancy. There is a large body of evidence specifically examining the influence of prematurity and VLBW. Low birth weight (LBW) is defined as infant weight that is below 2500 grams while VLBW is defined as infant weight that is below 1500 grams. Children who are born before the typical gestation period of 38-42 weeks are termed preterm infants. VLBW and preterm infants are typically placed in Neonatal Intensive Care Units (NICU) after birth and are separated from their parents almost immediately. Much of the current research on birth weight and attachment has investigated infants who are either VLBW or preterm.

Interestingly, a review reports that the effects of preterm birth and VLBW are inconsistent. (23, 28) Several studies find no difference in the attachment quality between full-term and preterm infants. (29-33) One study, looking specifically at VLBW infants, finds that birthweight does not associate with attachment quality. (34) Finally, studies looking at both preterm and VLBW infants find no significant difference in attachment quality when compared to full term infants. (14, 23, 35, 36) Only three studies report positive findings, including an association between preterm birth and increased frequency of insecure type attachment. (37) The other two studies compare VLBW children to regular birth weight children, finding positive results for the association between birthweight and attachment style. (38, 39)

Birth weight exists on a continuum. A slight decrease in birth weight, even if it is still considered to be in the normal range, has been shown to predict psychopathology. (40) There exists no research which examines the association between birth weight in the normal continuum and attachment. Due to the high correlation existing between prematurity and low birth weight, it has not been possible to distinguish the effect of growth from that of shortened gestational period.

Gene of Interest - DRD4 Gene

The dopamine D4 receptor (DRD4) is critical for the cognitive and emotional processes that are sub-served by neural circuits in the prefrontal cortex. (41) The DRD4 gene and its variants affect the dopamine receptor efficiency in the brain. It is well established that the DRD4 gene is associated with a child's ability to pay attention, with variants of the gene being associated with ADHD. (42-45) Since a child's attention is an integral part of how they may react to their mother and/or guardian, researchers have begun to look into the mechanisms of how the DRD4 gene can affect the attachment between a child and a mother. The association between DRD4 polymorphisms and disorganized attachment is of particular interest.

The DRD4 gene has two functional polymorphisms: a 48 base-pair variable number tandem repeat (VNTR) in Exon III and the -521C/T polymorphism in the promoter of the gene. In the VNTR, the common functional variants range from 2 to 11 copies (46), with the most common ones seen in humans being the 4-repeat (short) and the 7-repeat (long) alleles. (47)

One of the first studies evaluating the association between disorganized attachment and the DRD4 gene found that disorganized attachment is four times more frequent among infant carriers of at least one 7-repeat DRD4 allele. (48) Additionally, in the same sample, the -521 thymine allele is associated with disorganized attachment, with a ten-fold increase in the rate in association with the 7-repeat allele of DRD4. (49) However, the -521 thymine variant does not show a significant effect without the presence of the 7-repeat allele. (49)

Out of several attempts, few studies have replicated the significant association between the 7-repeat allele of the DRD4 gene and disorganized attachment. Since 2001, five studies have reported that the 7-repeat allele is not significantly associated with disorganized attachment in children. (50-54) Two studies report positive results, with one based on the same original sample. (48, 49, 55, 56) Collectively, with the exception of one study, all positive results showing association between the 7-repeat allele with disorganized attachment have been from the same sample of infants.

(48, 49, 56, 55)

Hypothesis

Given the inconsistent findings concerning the association between birth weight and the development of disorganized attachment between the child and mother, the current research seeks to confirm that there is no association between birth weight and disorganized attachment. Additionally, it is predicted that the DRD4 receptor gene polymorphisms would serve as a risk factor and have a positive main effect on the development of disorganized attachment between the child and mother.

Methods and Materials

The MAVAN Sample

The study sample comes from the Maternal Adversity Vulnerability and Neurodevelopment (MAVAN) project, an established cohort of mothers and children recruited between 2003 and 2009. (57) Please view the On-line Supplementary Methods Page for a detailed description of the MAVAN project.

Inclusion / Exclusion Criteria

Eligibility criteria included mother's age ≥ 18 years at the expected date of delivery, singleton gestation, and babies born at 37 weeks or longer of gestational age. We excluded women with severe chronic illness, placenta previa, a history of incompetent cervix diagnosed in a previous pregnancy, or impending delivery of an infant affected by a major anomaly.

Procedure

We followed the women during pregnancy. Birth outcomes were assessed at time of delivery. Mothers and their child were seen at 6, 12, 18, and 24 months and yearly subsequently.

Measures (Independent)

Birth weight: Adjusted using Canadian normative data.

Genotype: We coded DRD4 as 7-repeat vs. other genotypes from oral-buccal swab samples.

Measures (Dependent)

Attachment Style: We administered the modified separation-reunion procedure as previously described for preschool-age children. Administration occurred at 36 months, from which Disorganized classification (D) was obtained. (58)

Measures (Adjustment - Covariates)

Child: gender

Maternal: We obtained maternal education from a prenatal questionnaire and trichotomized (Table 1). (59)

Analysis

We present descriptive data for child and maternal variables. Univariate analyses examined the association between predictors, covariates, and disorganized attachment. We conducted a logistic regression model to test for the independent effects of the predictors.

Results

Descriptive

There was an almost equal distribution of boys and girls. For DRD4, 34.6% were carriers of at least one 7-repeat allele, while 65.4% were not. For disorganized attachment, 22.9% were categorized as disorganized, while 77.1% were not. The distribution of mothers included 15.6% with a high school degree or a partial college education, 32.0% with a college degree or some university education, and 52.4% with at least a university degree (Table 1).

Covariates

Males and females did not differ in their distributions of disorganized attachment, (χ^2 (DF = 1, N = 231) = 0.557, p = .455) (Table 2). The frequency of disorganized attachment in children differed according to their mother's education status. (χ^2 (DF = 2, N = 231) = 18.99, p = .000).

Environmental Factor of Interest – Birth Weight

Birth weight was not significantly associated with the development of disorganized attachment, ((DF = 1, N = 231) = 0.113, p = 0.738) (Table 2).

Gene of Interest - DRD4 Gene

The genotype was significantly associated with disorganized attachment, (χ^2 (DF = 1, N = 231) = 9.47, p = 0.02) (Table 2). Children without the 7-repeat allele were significantly associated with the development of disorganized attachment (Fig. 1). Children with the 7-repeat allele were 0.31 times as likely to develop disorganized attachment style when compared to children without the 7-repeat allele (Table 3).

Adjusted Analyses

Given that maternal education was found to be a significant covariate, a Logistic Regression Test was used to assess whether the associations were independent of the covariates. Birth weight remained unassociated with the development of disorganized attachment, (b = -0.002, p = 0.998) (Table 3). On the other hand, DRD4 genotype was still significantly associated with the development of disorganized attachment, (b = -1.120, p = 0.004).

Discussion

Birth weight did not have a significant effect on attachment type. This result is consistent with the majority of prior research on preterm/VLBW and attachment. It should be noted that one key distinction of our study is that birth weight percentile was used instead of length of term (preterm) or birth weight (VLBW). Prior literature has been limited by an inability to determine whether the birth weight of recorded infants was small due to actual low birth weight or simply because of prematurity. To ensure results were clear in the present study, infants who were born prior to 37 weeks of gestational age were excluded from the MAVAN sample.

Consistent with our second hypothesis, we found the DRD4 7-repeat polymorphism to be significantly associated with disorganized attachment. Contrary to expectations, results indicate that children with the DRD4 7-repeat allele are less likely to have disorganized attachment than children without the DRD4 7-repeat allele. Although statistical significance was achieved, the direction of the association was unexpected and opposite to our originally hypothesised relationship.

Our finding that children with the DRD4 7-repeat allele were less likely to have disorganized attachment is consistent with only one study. (55) In a sample including children with maltreatment, child maltreatment was found to interact with the DRD4 genotype to predict disorganized attachment. The researchers found that at 12 months of age, the maltreated group who received experimental intervention (but not the maltreated children who did not receive intervention nor the normal control group)

showed an association between DRD4 genotype and disorganized attachment. They found that children in this group who had the 7-repeat allele were less likely to display disorganized attachment as only 17.5% of those showing disorganized attachment had the 7-repeat allele while 82.5% of those showing disorganized attachment lacked the 7-repeat allele. While a sample examining maltreated children is very different from the MAVAN sample, this does provide intriguing reinforcement of the results found in this study. It should be noted that the children tested in this group were tested again at 2 years of age, and the results were not replicated. We note this to be the only other example of the DRD4 7-repeat allele being negatively associated with disorganized attachment. However, complementary findings indicate the 7-repeat allele may act as a protective factor very early in infancy (1–2 months of age) suggesting that the DRD4 7-repeat allele is associated with an easy temperament and more adaptive behaviour. (60–62)

While much of the attention in prior research has been on the negative implications of the DRD4 7-repeat allele, very little focus has been on the positive aspects. Previous research commonly emphasizes the cumulative negative effects of specific “risk genes” and an adverse rearing environment, whereas potentially cumulative positive effects of the same risk genes interacting with positive rearing environments remain understudied. (54, 63) Accordingly, a theoretical construct of ‘differential susceptibility’ has been tested to examine whether children with the “risk genes” are more susceptible not only to the effects of adverse environments, but also the beneficial effects of supportive rearing. (64, 65) The DRD4 could potentially operate as one such susceptible gene with meta-analytic findings providing validation. (63, 66) For example, children with the DRD4 7-repeat allele exposed to unresponsive maternal care displayed more externalizing behaviour problems than children without the DRD4 7-repeat allele, but children with the DRD4 7-repeat allele exposed to responsive maternal care showed the lowest levels of externalizing problem behaviour. (54)

In some instances, it may be the case that the DRD4 7-repeat allele does indeed function as a protective factor. Considering the 7-repeat allele originated as a rare mutation whose frequency increased in human populations by positive selection, there has arisen increased speculation about its contribution to the evolution and adaptability of human development. (41, 67) Such a possibility supports the need to go beyond the common labelling of the DRD4 7-repeat allele as merely a risk factor for various internalizing and externalizing disorders. – it may well have protective properties.

Our results support that such an influence may exist between the DRD4 7-repeat allele and disorganized attachment. It is well established that the 7-repeat allele is associated with ADHD and decreased attention. (42–45) Lower dopaminergic signalling impedes negative feedback-based learning and is associated with stronger preference for immediate reinforcers. (68, 69) However, this may be advantageous or disadvantageous depending on specific environmental characteristics. (70) In our study sample, infants with the 7-repeat allele were less attentive to their environment and the parenting style of their caregivers during early years; this genotype may thus serve as a protective factor. However, conclusions as to why this might occur cannot be deduced until maternal sensitivity is measured alongside this genotype.

From the contradictory results accumulated for both birth weight and DRD4 genotype in terms of attachment, it is clear that several factors are at play. It is important that gene x environment interaction studies with various different factors are conducted. Rates of disorganized attachment are high when compared to other endophenotypes, reaching a frequency of one in six children in low-risk populations. Having significant numbers of a population exhibit endophenotypes for any type of psychopathology is not only a psychological problem for the individual, but also poses a significant social and economic challenge. The burden of assisting those with depression and other psychopathologies put strain on our social service and health care resources. Such challenges can be addressed by study designs which consider genetic influences, neural pathways and environmental factors potentially implicated in the mother-child relationship.

Limitations

The subsample used to test the hypotheses reflects a significant drop-out rate. Not all of the children have been genotyped or tested for attachment yet. In addition, attachment style and disorganized attachment is a very complex psychological dynamic that is likely affected by multiple different genetic and environmental factors. Several other genetic and environmental factors from both the mother’s and the child’s experience could have an effect with the development of disorganized attachment. When compared to other genetic studies, the MAVAN has a relatively smaller number of participants. Our power, however, is strengthened by the accuracy of our genotyping method, precise functional sub-categorization of the DRD4 allele (the presence of at least one 7-repeat allele), and the experimental measure of attachment. (71) Finally, we only examined a monogenic model. There is growing evidence for the interaction of genes (GxG) and specifically the association of DRD4 with 5HTTLPR but also with MAO. One interesting possibility, for example, would involve norepinephrine-related genes, given that norepinephrine acts in concert with dopamine to influence attentional processes and likely attachment disorganization. (72)

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Figures and Tables

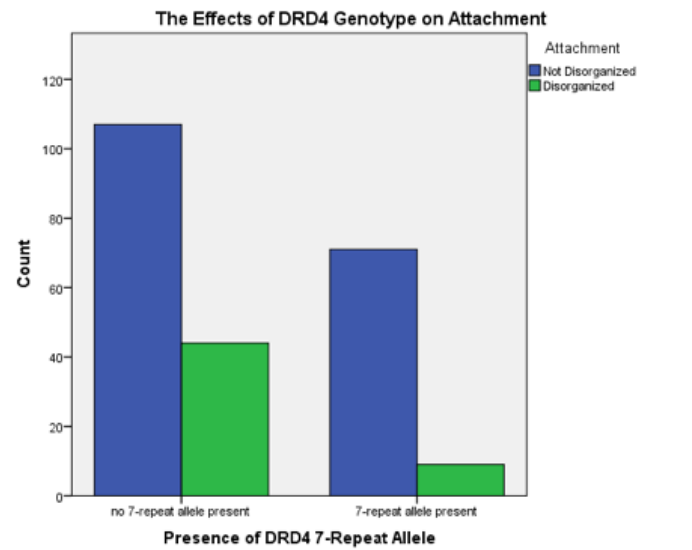


Figure 1. The Effects of DRD4 Genotype on Attachment. The darker bars represent not disorganized attachment, while the lighter colour represents disorganized attachment.

Variables	Montreal and Hamilton (N = 231) N (%)
Mothers	
Education	
High school or partial college education	36(15.6%)
Completed college degree or partial university education	74(32.0%)
University graduate degree or more	121(52.4%)
Children	
Gender:	
Male	115(49.8%)
Female	116 (50.2%)
Birth Weight (grams):	Mean: 3353.20 Std. Deviation: 467.157
Birth Weight (percentile):	Mean: 45.49 Std. Deviation: 27.987
Birth Weight:	
Low Birth Weight (< 2500 g)	6 (2.60%)
> Low Birth Weight (> 2500 g)	225 (97.4%)
Genotype DRD4:	
Presence of at least one 7-repeat allele	80(34.6%)
No 7-repeat allele	151(65.4%)
Attachment:	
Disorganized	53(22.9%)
Not disorganized	178(77.1%)

Table 1. Demographic Characteristics of subjects from MAVAN

	Mean	Disorganized N (%)	X ²	T-test	DF	Sig
Birth weight (percentile)	45.49			0.113	229	0.738
Disorganized	45.79					
Not disorganized	44.47					
DRD4			9.47		1	0.02
7-Repeat Allele		9 (3.9%)				
Other Allele		44 (19%)				
Gender			0.557		1	0.455
Male		29 (12.6 %)				
Female		24 (10.4 %)				
Maternal education			18.99		2	0.000
High school or partial College education		18 (7.80%)				
Completed college or partial University education		10 (4.30%)				
University graduate degree or more		25 (10.8%)				

Table 2. The prediction of Disorganized Attachment from birth weight and DRD4 – Univariate analysis

Predictors	B	SE	Exp(B)	Odds Ratio	Confidence Interval (95%)	Sig.
Birth weight (percentile)	-0.002	0.006	1.363			0.998
DRD4	-1.120	0.402	0.326	0.308	0.142-0.671	0.004
Covariates						
Maternal Education	-0.521	0.213	0.594			0.014
Gender	0.310	0.327	1.363	1.26	0.683-2.34	0.344

Table 3. The prediction of Disorganized Attachment from birth weight and DRD4 using a logistic regression model

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Keywords

Cancer immunotherapy: a cancer treatment that relies on coaxing the immune system to selectively target cancerous cells

Cytotoxic T lymphocyte: a type of white blood cell that identifies infected or cancerous cells; releases enzymes to destroy the target cell

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Immune Response Regulation has Therapeutic Potential in the Treatment of Cancer

Abstract

Background: Depending on their path of differentiation, immune cells can have opposing roles in tumour progression. As a result, during growth, tumours undergo selective pressure to produce immunosuppressive factors that contribute to tumour growth, angiogenesis, and metastasis. This review discusses the contribution of different macrophages and T cells to tumour progression, as well as their role in current cancer immunotherapies.

Methods: We searched for articles online through McGill Library with search terms including the names of different immune cells along with “polarity”, “tumour progression”, or “cancer immunotherapy”. Cancer therapies “CTLA-4 blockade”, “Ipilimumab”, “adoptive cell transfer”, and “PD1 inhibition” were also used as search terms.

Summary: Depending on the cell types involved, crosstalk between different immune cells in the tumour stroma can contribute to either the development or the inhibition of tumour growth. Certain therapies such as adoptive cytotoxic T lymphocyte (CTLs) transfer and CTLA-4 & PD1 inhibition work by enhancing CTL tumoricidal responses, and have produced durable responses in a small but significant group of patients. Other therapies work by skewing the phenotype of tumour associated macrophages from pro-tumorigenic to anti-tumorigenic. However, disrupting the balance between immune cell functions risks triggering inflammatory disorders such as autoimmunity. Therefore, future directions in cancer immunotherapy include targeting potential responders and restricting therapeutic mechanisms to the tumour microenvironment.

Introduction

Inflammation at the tumour site is thought to enable several hallmarks of cancer such as sustained tumour cell proliferation and survival as well as angiogenesis and metastasis. (1) For instance, several bone marrow-derived cells such as macrophages, neutrophils, mast cells and myeloid progenitors contribute importantly to the onset and maintenance of angiogenesis, the formation of vasculature required to sustain tumour growth. Furthermore, these cells have been found to promote metastasis by producing matrix-degrading enzymes and by stimulating the epithelial-to-mesenchymal transition involved in tumour cell invasion. (1) Not only do tumour cells undergo selective pressure to populate their microenvironment with immune cells that foster their progression, they also undergo pressure to evade destruction by tumoricidal leukocytes, such as cytotoxic T lymphocytes (CTLs), natural killer cells, and specific subsets of macrophages and neutrophils. (1) Tumour immune evasion is thought to rely on reduced antigen presentation at the tumour cell surface, on the production of immunosuppressive molecules, and on the recruitment of immune cells that inhibit the activity of anti-tumour leukocytes. The interaction of tumours with immune cells in their microenvironment has warranted the development of therapies aimed at stimulating tumour-cytotoxic immune cells and at inhibiting the tumour-leukocyte interactions that promote cancer progression. This review focuses on the role of certain T cells and macrophages in tumour progression and therapy, as these cell types are among the most studied in the context of tumour immunology. However, several other immune cells and non-immune cells have important roles in tumour progression. To date, adoptive transfer of tumour-specific T cells as well as T cell checkpoint inhibition have resulted in durable responses to cancer, including some complete responses lasting several

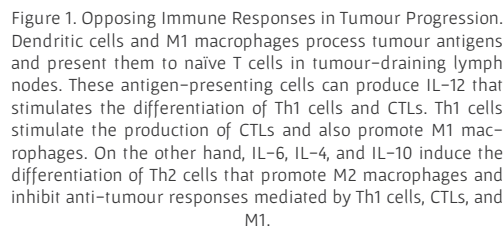
years, and have increased overall survival. Other therapeutic strategies involve inhibiting the recruitment, differentiation, or function of immune cells, such as regulatory T cells and M2-type macrophages that inhibit anti-tumour immune responses and foster tumour immune evasion and progression.

Cytotoxic Lymphocytes in Cancer Therapy

CTLs are a subset of CD8+ T cells in which every clone harbours a unique antigen specificity. CTLs specific to a tumour-associated antigen can kill tumour cells presenting the antigen with Major Histocompatibility Complex (MHC) class I at their surface. The cytotoxicity of a CTL requires the binding of its T cell receptor (TCR) to this peptide-MHC class I complex on the tumour cell surface. (2) CTLs can then trigger the apoptosis of a tumor cell via their expression of the ligand for the Fas receptor found on the tumor cell membrane. CTLs also express perforin, which forms holes in the tumour cell membrane through which the CTL injects granzymes. These granzymes activate the caspases that drive apoptosis. IFN γ produced by CTLs upregulates Fas expression and is therefore important for inducing tumor cell apoptosis. (2)

In order to mount an anti-tumour immune response, antigen-presenting cells such as macrophages or dendritic cells must take up tumour antigens at the tumour site and travel to tumour-draining lymph nodes. Once at the lymph nodes, they activate CD4+ and CD8+ T cells specific to the antigen by presenting the antigen-MHC complex to the T cell receptor and by presenting co-stimulatory ligands to the T cell. CD4+ T cells are called T

ptides are encoded by genes normally expressed in male germ cells and are not usually presented to T cells, since male germ cells do not express MHC molecules. Some tumours activate the expression of antigens such as melanoma associated antigen (MAGE) or NY-ESO-1 in oesophageal cancer, melanoma, breast cancer, prostate cancer, bladder cancer, or non-small-cell lung carcinoma. (2, 9) However, these proteins are also expressed in the thymic cortex, so T cells specific to these antigens have low affinity for the peptides due to the pressures of negative selection. Other tumour-restricted antigens are derived from mutant proteins, most often from point mutations such as in Ras or p53. (2) For instance, T cells specific to a K-Ras point mutant epitope were isolated from pancreatic tumours. (10) T cells specific to point mutated Ras did not recognize wild type Ras. (11) Other tumour antigens are processed from fusion proteins, such as BCR-Abl, or proteins that undergo defective post-translational modifications such as underglycosylated mucin (MUC-1) in breast and pancreatic cancers. Finally, viral oncogenes can also be expressed as tumour antigens. Not all abnormal proteins can bind MHC molecules and be presented to T cells. However, it is now widely accepted that most tumours are immunogenic either via their surface overexpression of shared antigens or their presentation of modified self or viral peptides.



It is not clear whether mutant proteins generate more immunogenic epitopes than non-mutated self-peptides. Further evidence suggests that the context of antigen presentation is more important than the degree of selfness or non-selfness of tumour antigens. (8) For instance, infection with a tumour-associated virus triggers acute inflammatory responses that activate T cells to more effectively destroy infected and transformed cells. This is supported by the higher incidence of viral-associated cancers in immunodeficient individuals. On the other hand, chronic antigen presentation and inflammation seem to promote immune tolerance and T cell anergy. Adenoviral DNA vectors encoding tumour antigens are currently being used to stimulate the Th1/CTL response against self-antigens. For instance, a study by Naveh et al. (2013) investigated the transduction of dendritic cells with a replication-deficient adenoviral vector encoding three melanoma antigens: MART-1, tyrosinase melanocyte antigens, and MAGE-A6 cancer-testis antigen. (12) Viral DNA stimulates dendritic cells to present antigen to T cells and secrete cytokines such as IL-12 that go on to promote the differentiation of Th1 cells and CTLs. This method is particularly effective at activating tumour specific CTLs since the viral vector promotes antigen presentation by dendritic cells in complex with MHC class I molecules. Acute inflammatory responses triggered by dendritic cells in response to viral DNA may be a method of preventing tolerance and promoting stronger T cell responses against tumour antigens.

A barrier to effective anti-tumour CTL responses involves the ability of tumour cells to escape detection by downregulating components of the complex cellular machinery responsible for the processing and presentation of endogenous peptides at their surface. These components include the catalytic proteasome subunits LMP2, LMP7, and LMP10 that generate peptide fragments; the transporters TAP1 and TAP2 that carry these peptides into the endoplasmic reticulum; ERAP1 that trims the peptides for loading onto the MHC class I complex; and ER chaperones such as tapasin that enable the assembly of the MHC-peptide complex. Human bladder cancer, melanoma, and colorectal carcinoma specimens having reduced MHC class I surface expression were found to have downregulated one or more of these components. (13) Loss of LMP7, TAP1, and ERAP1 were associated with reduced overall survival in human cervical cancer. (14) Therefore, targeting the mechanisms by which tumour cells downregulate peptide-MHC class I surface expression has strong potential to contribute to effective cancer immunotherapy. Other mechanisms by which tumour cells avoid destruction by CTLs include the expression of inhibitory ligands such as PDL1, the production of factors such as IL-10 and TGF- β that inhibit CTL function, and the recruitment of regulatory T cells that also block CTL activity.

Tumours may escape destruction by CTLs by promoting and recruiting another CD4+ T cell subset called regulatory T cells (Treg). Treg cells

inhibit the activation of lymph node T cells that bear the same antigen specificity as that of the Treg cell itself. They can therefore prevent the development of T cells that will target tumours. This inhibition results from a receptor called CTLA-4 on the membrane of Treg cells that competes with greater affinity compared to the T cell activating receptor CD28 for its ligand B7 on antigen-presenting cells. (15) The activation of CD28 via its engagement with B7 is essential for T cell proliferation upon TCR-antigen interaction. CD28-mediated signalling induces T cell production of the cytokine IL-2 as well as the assembly of the IL-2 receptor. This process enables the necessary IL-2 autocrine signalling for T cell survival and proliferation. (2) Furthermore, Treg cells have a greater capacity to bind IL-2 and may therefore sequester it from other T cells. (2) Treg cells can also kill dendritic cells bearing the same antigen specificity. (16) IL-10 produced by Treg cells in the tumour stroma inhibits the maturation and antigen presenting activity of dendritic cells and macrophages. (17) Therefore, Treg cells inhibit the activation and expansion of tumour-specific Th1 cells and CTLs in lymph nodes both by blocking the presentation of tumour-antigens to T cells, and by blocking the CD28 and IL-2 signalling necessary for T cell survival and proliferation. At the site of the tumour, Treg cells can inhibit CTL activity via membrane-bound or secreted transforming growth factor beta (TGF- β). (18) Treg cells have been shown to downregulate INF γ and perforin production by CTLs via direct cell-cell interactions. (19) Treg cells are induced by TGF- β ; the presence of elevated levels of TGF- β upon the presentation of a tumour antigen to naïve CD4 $^{+}$ T cells in tumour-draining lymph nodes may promote their differentiation into Treg cells. Production of IL-10 and TGF- β by human cervical cancer cells is associated with decreased CTL function, inhibition of type 1 T cell polarity, and tumour invasiveness. (4) Studies relating to breast and ovarian cancer report increased Treg infiltration with disease stage in association with reduced relapse-free survival, and reduced overall survival. (20, 21)

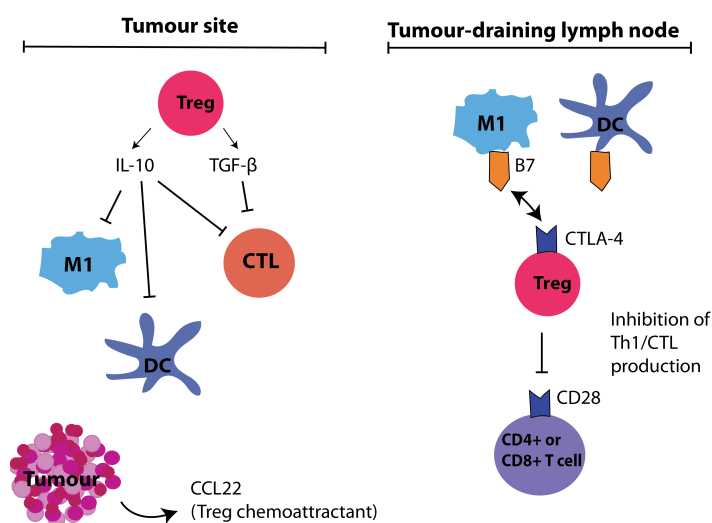


Figure 2. Regulator T Cells Inhibit Anti-Tumour Immunity. At the tumour site, Treg cells secrete IL-10 that inhibits M1 macrophages, dendritic cells, and CTLs. Membrane-bound TGF- β on Treg cells also inhibits CTL activity. Tumours produce the Treg chemoattractant CCL22. In tumour-draining lymph nodes, CTLA-4 on Treg cells sequesters B7 ligand from its receptor CD28 on naïve T cells, inhibiting their activation and proliferation into Th1 cells and CTLs.

Tumour cells may also evolve the ability to recruit Treg cells. In a study by Benevides et al. (2013), Treg infiltration was observed in breast invasive ductal carcinomas (IDC) as opposed to healthy controls, and was correlated with IDC elevated expression of CCL22, a chemokine that attracts Treg cells. Blocking the interaction between CCL22 and its receptor CCR4 on Treg cells using a CCR4 agonist has therapeutic potential by inhibiting the recruitment of Treg cells to lymphoid organs and to the tumour site. (22) Studies in mice have shown that the administration of tumour antigens as vaccines along with the administration of a CCR4 small molecule antagonist enhances the production of tumour-specific CTLs. (23) Tumour

cells and their associated macrophages were shown to secrete CCL22 in ovarian carcinoma. (20) Therefore, tumours may have the ability to promote tolerance to any tumour antigen by producing factors that inhibit CTL-mediated responses and by promoting the production and recruitment of Treg cells.

Adoptive T Cell Transfer

Some of the most striking advances in cancer immunotherapy conducted in melanoma treatment were inspired by cases of spontaneous regression that encouraged the trial of T cell-targeted immunotherapies in metastatic melanoma patients. The most durable responses have been reported for immunotherapies such as adoptive T cell transfer and the inhibition of the T cell receptors CTLA-4 and PD1 that promote CTL anergy.

Adoptive T cell transfer therapy involves culturing a patient's tumour-infiltrating T cells ex-vivo in several cultures established from single cell suspensions and then selecting for Th1 cells and CTLs that are specific to the patient's tumour antigens. This is done by co-culturing the T cells with tumour cells and selecting for IFN γ production. The T cells are cultured in the presence of IL-2 to stimulate their proliferation. The selected T cells are then injected into the patient's circulation.

Adoptive cell transfer (ACT) has shown promising results from three clinical trials on metastatic melanoma patients. (6) Tumour-infiltrating T cells from metastatic lesions were cultured in the presence of IL-2 and tested for tumour specificity via the methods described above. They were injected back into patients along with IL-2 after lymphodepleting non-myeloablative (NMA) chemotherapy. Twenty-two percent of patients experienced complete responses, of which 93% had disease free survival at five years. The five-year survival rate of the entire cohort was 29% compared to about 5% following the standard of care or IL-2 therapy. Patients that received lymphodepleting chemotherapy followed by ACT had an objective response (OR) rate of 48%, whereas patients who also received low dose or high dose full-body irradiation prior to ACT had an OR rate of 52% and 72%, respectively. The complete response rates for these three cohorts were 12%, 20%, and 40%. The proportion of complete responses and their durability is much higher than in BRAF inhibitor therapies, which have a 6% complete response rate, and decarbazine, which has a 1% complete response rate. (6) Interestingly, patients who had previously received CTLA-4 blockade therapy showed an increased overall survival rate.

Non-lytic doses of irradiation (up to 20 Gy) enhance the susceptibility of cancer cells to cytotoxic T cell-mediated killing. They promote the upregulation of Fas expression at the tumour cell surface, as well as the expression of MHC class I and intracellular adhesion molecule 1 (ICAM-1), which binds receptors on T cells. (24) The increased susceptibility to CTL attack as a result of irradiation may explain in part the higher OR rates seen in patients having undergone irradiation.

Other studies have reported the synergy and potential clinical benefit of combining adoptive T cell transfer with the BRAFV600E inhibitor Vemurafenib in melanoma patients. BRAFV600E inhibition upregulates the surface expression of melanocyte-differentiation antigens MART, gp100, and tyrosinase with MHC class I complexes and therefore enhances the recognition of melanoma cells by T cells having low affinity for these antigens. (25) MAPK inhibition could also enhance CTL recognition of other cancer cell types, but it is important that the inhibitor be specific to a mutant upstream kinase only found in transformed cells to avoid destruction of normal cells by CTLs. The kinase inhibition also sensitizes melanoma cells to induced apoptosis by CTLs. (25) Furthermore, it has been shown that mutant BRAF signalling upregulates the production of IL-1 by melanoma cells, which increases tumour-associated fibroblast expression of ligands for the inhibitory receptor PD1 on T cells. Activation of the PD1 death receptor on CTLs inhibits their activity and can lead to their apoptosis. Melanoma cells from patients undergoing Vemurafenib treatment express less IL-1 than before treatment, and their tumour-associated fibroblasts are less able to inhibit T cell cytotoxicity in vitro. (26) Clinical trials are currently evaluating the safety and efficacy of combining Vemurafenib and

Sipuleucel-T (Provenge) is a form of adoptive cell transfer therapy approved for metastatic castration-resistant prostate cancer. The therapy target expanding T cells specific to the prostate antigen, prostatic acid phosphatase, that is overexpressed in 95% of prostate cancers. (27) The method involves isolating a patient's peripheral mononucleated blood cells, which include T cells, macrophages, and dendritic cells. The cells are cultured in vitro with a fusion protein of prostatic acid phosphatase (PAP) and granulocyte-macrophage colony-stimulating factor. The fusion protein promotes the uptake of the tumour antigen by macrophages and dendritic cells and its presentation to T cells. A double blind randomized phase III trial was conducted by Kantoff et al. (2010) in which 512 patients were given either three injections of leukocytes cultured with the fusion protein or leukocytes cultured with no antigen over two weeks. (28) The study reports that 46 out of 63 patients from Sipuleucel-T group (73%) had proliferative T cell responses to the fusion antigen at six weeks post-infusion. Median survival was increased by 4.1 months, and the probability of survival at 36 months was 32% for the group receiving Sipuleucel-T, compared to 23% for the placebo group. However, this survival increase is modest compared to the ACT trials conducted in melanoma patients.

CTLA-4 blockade: Ipilimumab

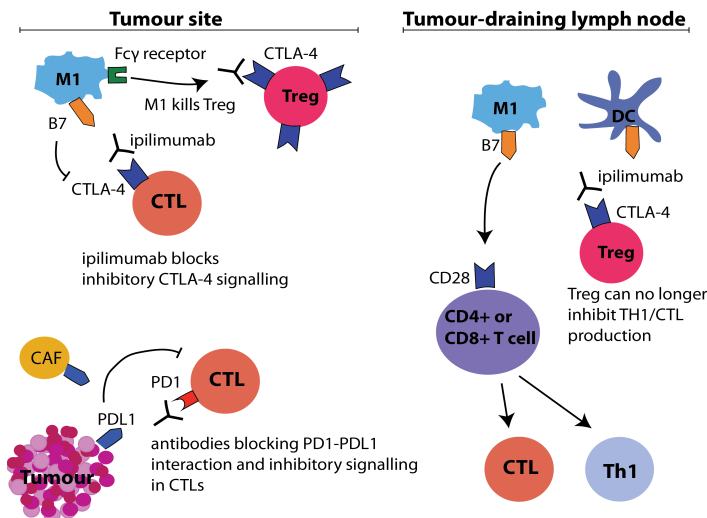


Figure 3. T Cell Checkpoint Inhibition. At the tumour site, Ipilimumab inhibits the interaction of the inhibitory receptor CTLA-4 on CTLs with its ligand B7 on other immune cells. Ipilimumab may also enable the depletion of Treg cells at the tumour site by binding Treg CTLA-4 and mediating the destruction of Treg cells by macrophages bearing Fcγ receptors that interact with the constant region of Ipilimumab. Other antibodies inhibit the interaction between the inhibitory receptor PD1 on CTLs and its ligand on tumour cells and on cancer-associated fibroblasts (CAF).

Other cancer immunotherapies enhance CTL killing of tumour cells by blocking their inhibitory receptors, CTLA-4 and PD1. (Fig. 3) Ipilimumab is a fully human, monoclonal IgG antibody against CTLA-4, an inhibitory receptor expressed on activated T cells. This then induces T cell anergy upon interaction with its ligand, B7, on macrophages or dendritic cells at the tumour site. Ipilimumab can also block Treg inhibition of CTL production in lymph nodes by binding CTLA-4 on Treg cells and inhibiting its sequestration of activating ligands away from T cells. Furthermore, recent evidence suggests that Ipilimumab depletes Treg cells at the tumor site by promoting the killing of Treg cells. Treg cells are killed by macrophages located in the tumor stroma that bind the constant region of the antibody via their Fcγ receptor. (29) It is thought that effector T cells are not targeted by macrophage cytotoxic activity because they express much less CTLA-4

than Treg cells. Therefore, Ipilimumab enhances the production and function of CTLs by blocking inhibitory interactions at the tumour site and in lymph nodes. Ipilimumab was approved in 2011 for the treatment of metastatic melanoma. Clinical data indicates that patients receiving Ipilimumab alone had an OR rate of 13%, whereas patients who received IL-2 had an OR rate of 25% with a 17% rate of complete responses, and higher overall survival. (30) IL-2 is known to promote CTL survival, proliferation, and secretion of IFNγ. Toxicity of CTLA-4 inhibition involves immune-related adverse effects (IRAEs) due to the role of CTLA-4 in regulating inflammation and undesirable autoimmune responses. Grade 3 or 4 IRAEs were observed in 10% to 15% of patients receiving Ipilimumab. Treatment with Ipilimumab improved overall survival with a 34% reduction in probability of death compared to tumour vaccines, and 26% compared to IL-2 therapy alone. (31) Responses to Ipilimumab treatment are strikingly durable and associated with significant increases in overall survival. Results from three phase II trials indicate that previously treated patients receiving Ipilimumab have 4 year survival rates of 20% to 28%, and treatment naïve patients have 4 year survival rates of 38% to 50%. (32) In a phase III clinical trial, 1 and 2 year survival rates for patients receiving Ipilimumab were 46% and 24% compared to 25% and 14% in the gp100 vaccine control group. (33) The safety and efficacy of Ipilimumab therapy is currently being evaluated in trials for other cancers such as non-small-cell lung cancer and prostate cancer.

PD1 blockade

Another T cell checkpoint inhibition therapy that has revealed striking clinical benefits in some patients is PD1 blockade. Tumour cells can inhibit CTL cytotoxic activity by expressing ligands for the PD1 programmed death receptor on CTLs. (34) Tumour PD1-ligand (PDL1) interaction with PD1 on T cells inhibits CD8+ T cell proliferation and production of cytokines such as IFNγ, and can also lead to T cell apoptosis. A phase I clinical trial conducted by Topalian et al. (2012) evaluated the safety and response to treatment with an anti-PD1 antibody. OR rates were 18% for non-small-cell lung cancer patients, 28% for melanoma patients, and 27% for renal cell carcinoma patients. Grade 3 or 4 drug-related adverse effects were reported in 14% of patients, along with 3 deaths from pneumonitis out of 296 patients, of which two were patients with non-small-cell lung cancer and one was a patient with colorectal cancer. Ninety percent of patients had received three or more infusions of Ipilimumab 12 weeks before enrolling in the trial. (35) In a follow up study on 39 patients treated with anti-PD1, three patients experienced durable objective responses. (36) One metastatic colorectal adenoma patient had been refractory to multiple chemotherapy regimens before anti-PD1 therapy and developed a complete and durable response with no recurrence at the time of data collection three years later. A patient with renal metastatic carcinoma also experienced a complete response to anti-PD1 ongoing at the latest follow up four years off therapy. A third patient had melanoma, and prolonged administration of anti-PD1 stabilized disease resulted in a partial response lasting several years. Thus, there exists a correlation between tumour cell expression of PDL1 and response to anti-PD1 therapy.

Another anti-PD1 monoclonal antibody, Lambrolizumab, was evaluated in a phase I trial on 135 patients with metastatic melanoma. (37) The highest dose cohort (10mg/kg) showed a 52% OR rate, and overall response in the whole dose escalation cohort was 38%. There were grade 3 or 4 drug-related adverse effects in 13% of patients, and most responses were durable at follow up after 11 months.

Adoptive T cell therapy and checkpoint inhibitors against PD1 and CTLA-4 have resulted in durable complete responses in a small but significant proportion of patients, and have shown improvements in overall survival. There is a great need to determine the characteristics of patients that will respond to these therapies in order to better target these treatments. The limitations of adoptive T cell therapy relative to small molecule inhibitors are its labour-intensive requirements and cost. Given the success of Ipilimumab and anti-PD1, small molecule inhibitors which prevent restrictions to the production and activity of tumour specific T cells appear as a more feasible and effective cancer immunotherapy.

However, stimulating CTL tumour-specific responses by expanding these cells or by inhibiting their checkpoints may not be sufficient to mount an effective anti-tumour response. For instance, this therapeutic approach may be ineffective against tumours that have downregulated antigen presentation at their cell surface and that can escape recognition by CTLs. Another approach is to skew the tumour microenvironment from pro-tumorigenic to anti-tumorigenic. This is achieved by countering the tumour's ability to promote the differentiation and recruitment of immune cells which otherwise contribute to tumour progression. As previously discussed, different subsets of infiltrating T cells have opposing effects on tumour progression. Th1 cells play a significant role in promoting CTL and M1 mediated tumour cytotoxic responses, and their activity is opposed by Th2 cells and Treg cells. Th17 cells represent another subset of CD4+ T helper cells found in the tumour microenvironment; however, their role in tumour progression remains controversial. Their production of the cytokine IL-17 has been associated with reduced disease-free survival of breast cancer patients after chemotherapy. (38) IL-17 promotes the production of IL-6 by tumor cells and results in IL-6 autocrine signaling that promotes tumour cell proliferation, survival, angiogenesis, and invasion. (39-44) Some studies report the production of INF γ by Th17 cells and suggest plasticity in the Th17 cytokine profile. (45, 46) However, Th17 production of INF γ is driven by the transcription factor T-bet that is responsible for Th1 differentiation. Therefore, this transition from IL-17 to INF γ production may result from a transition from the Th17 to the Th1 phenotype. Indeed, this transition occurs naturally in inflammatory responses to reduce the strong inflammatory effects of IL-17. (46) Furthermore, the different subsets of T helper cells have important effects on tumour progression through their crosstalk with other immune cells. The crosstalk between T helper cells and macrophages has an important role in determining whether a tumour-cytotoxic response will be initiated or shut down. These interactions can be generally divided into type 1 and type 2 responses. The type 1 response consists of a positive feedback loop between Th1 cells and M1 macrophages. Th1 cells stimulate the differentiation of M1 macrophages that are tumour cytotoxic and that present antigen to T cells and stimulate their differentiation into Th1 cells and CTLs. (2) The type 2 response involves the interaction between Th2 cells and pro-tumorigenic M2 macrophages, whereby Th2 cells promote M2 differentiation. (47) M2 macrophages foster tumour growth, angiogenesis and invasion. (48) They are also immunosuppressive and interact with Treg cells. Importantly, the phenotype of T helper cells and macrophages is plastic and depends on the signals they receive. (2) Therefore, these response axes can be skewed by the tumour toward a type 2 response, or skewed by therapeutic intervention toward a type 1 response. The following section will discuss these responses in more detail, as well as the therapies aimed at their manipulation.

Macrophages in cancer progression and therapy

Similarly to T cells, macrophages can have pro-tumorigenic or anti-tumorigenic properties depending on their path of differentiation. (Fig. 4) Macrophages are generally classified into two effector types: M1 and M2. Often, macrophages in the tumour microenvironment are polarized toward M2 functions and contribute to tumour growth, angiogenesis, and metastasis. (49) These macrophages are often called tumour-associated macrophages (TAMs). A study following surgically treated renal cell carcinoma patients observed that high proportions of M2 and low proportions of M1 macrophages in tumours were associated with reduced survival, whereas higher M1 presence was associated with increased survival. (50) INF γ produced by Th1 cells, NK cells, or CTLs promotes the differentiation of M1 macrophages that can kill tumour cells via nitric oxide and tumour necrosis factor (TNF) production. (51) Th1 cells are crucial for the production of M1 macrophages and CTLs. M1 macrophages express elevated levels of MHC class II molecules and produce IL-12, enabling them to present antigen to CD4+ T cells and induce their differentiation into activated Th1 cells that will go on to stimulate a CTL response. Th1 cells, M1 macrophages, and CTLs can, taken together, positively regulate each other and mount an anti-tumour response. IL-4 produced by Th2 cells promotes immunosuppressive M2 macrophages that have reduced antigen presentation ability. M2 macrophages secrete IL-10 that stimulates PDL1 expression and suppresses CTL activity, in addition to inhibiting dendritic

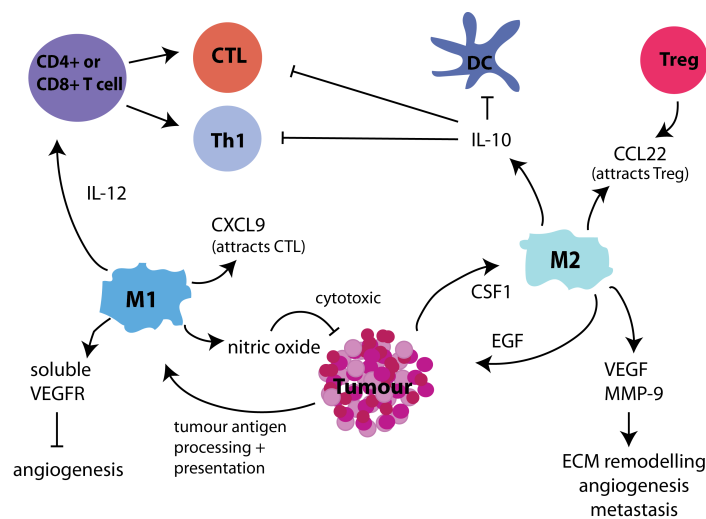


Figure 4. Opposing Roles of Macrophages in Tumour Progression. M1 macrophages inhibit tumour progression by promoting Th1 and CTL responses, inhibiting angiogenesis, and producing tumour-cytotoxic nitric oxide. M2 macrophages inhibit Th1 and CTL mediated anti-tumour responses and promote angiogenesis and metastasis. Tumour cells produce CSF1 and M2 cells produce EGF in a paracrine loop. CSF1 attracts macrophages and promotes an M2 phenotype. EGF promotes tumour progression and further production of CSF1.

cell antigen presentation and IL-12 production. M1 macrophages produce the chemokine CXCL9 that attracts CTLs whereas M2 macrophages produce CCL22 that attracts Treg cells. (20)

The polarity of macrophages is associated with the opposing activities of the transcription factors NF- κ B and STAT3. NF- κ B activates the production of IL-12, and the switch from active to inactive NF- κ B in macrophages appears to be central to tumour malignancy. (52, 53) IL-10, Vascular endothelial growth factor (VEGF), and IL-6 are activators of STAT3 in macrophages, and STAT3 induces the production of IL-10 and down-regulates IL-12. (54) In this mechanism, STAT3 activity is important for immune tolerance to tumours; inhibition of STAT3 was shown to enable the activation of T cells. (55) Several studies suggest that the dichotomy of M1/M2 macrophages is not clear cut and that M1 and M2 are the classified phenotypes at both ends of a spectrum of gene expression in macrophages. (56) The properties of macrophages in tumour progression may depend on their relative pro-tumorigenic and anti-tumorigenic activity, and there is substantial evidence that tumours regulate these properties to their advantage.

Macrophages are recruited to hypoxic areas of tumours via their binding to VEGF, CSF-1, and MCP-1 chemoattractants produced by tumour cells in response to the activation of HIF-1 transcription factor. Several tumour cells and their associated stroma also acquire the ability to produce other macrophage chemoattractants such as CXCL12, CXCL-8, and CCL9. (48, 57) In these hypoxic areas of the tumour, upregulation of HIF-1 in macrophages results in the production of VEGF and FGF2 angiogenic factors involved in the angiogenic switch that is important for tumour growth and metastasis. (35, 41, 43-44) Macrophages at the tumour site also secrete proteases such as matrix metalloproteinases that degrade the extracellular matrix (ECM) surrounding tumours and enable vessel remodelling as well as tumour cell migration. Furthermore, ECM degradation releases growth factors and pro-angiogenic factors into the tumour microenvironment. M2 macrophages are associated with these angiogenic properties, and M1 macrophages can actually secrete soluble VEGF receptor that neutralizes VEGF. (58) Finally, when tumour cells extravasate, they induce tissue resident macrophages to produce Matrix metalloproteinase 9 (MMP-9), leading to the release of VEGF. The resulting tissue and vessel remodelling enables the establishment and growth of metastases.

CSF1 is an important macrophage chemoattractant and differentiation fac-

tor for tumour invasiveness. CSF1 secreted by tumours can skew the phenotype of surrounding macrophages from M1 to M2. (59, 60) Consistent with these findings, the over-expression of CSF1 in breast tumours and the high density of tumour-associated macrophages correlate with poor prognosis. (46, 48) Importantly, an EGF-CSF1 paracrine loop contributes to tumour growth and invasiveness. Macrophages recruited to the tumour site via CSF1 secrete EGF that, amongst other EGFR-signalling effects on tumour growth, induces their epithelial to mesenchymal transition and promotes further secretion of CSF1 by tumour cells. (48) Macrophages and tumour cells migrate together toward blood vessels, and macrophages facilitate the intravasation of tumour cells and can associate with metastatic cells in circulation. (49) Transgenic mice lacking CSF1 have delayed progression to invasive mammary carcinoma and a large reduction in the incidence of metastasis, whereas expression of CSF1 in mammary epithelium and increased macrophage tumour infiltration accelerates tumour progression and increases metastasis to the lung. (61)

These data underline the therapeutic potential of inhibiting CSF1 or its receptor. Clinical trials are currently evaluating CSF1/CSF1R blockade. A study by Strachan et al. (2013) evaluated the effect of blocking CSF1 signalling in murine cervical and mammary cancer models. They found that the turnover of macrophages in the tumour microenvironment is dependent on CSF1 therefore the inhibition of CSF1R signalling may be therapeutically effective in depleting them. Treatment with CSF1R or CSF1 inhibitors significantly decreased the number of tumour infiltrating macrophages and neoplasm size, and also resulted in increased tumour infiltration of CD8⁺ T cells. (60) Another study reported that TAMs had the ability to promote cancer stem cell traits via STAT3 signalling; and that efficacy of chemotherapy was increased when mice were treated with a CSF1R inhibitor, due to a decrease in STAT3-dependent chemoresistance. (62)

Another macrophage-targeted therapy involves skewing the phenotype of TAMs rather than depleting them. A study by Rolny et al. (2011) investigated the ability of an anti-angiogenic histidine-rich glycoprotein (HRG) to induce an M2 to M1 switch in tumour-associated macrophages in mice via its downregulation of PIGF growth factor. Expression of factors produced by M2, such as IL-10 and CCL22, was reduced and expression of M1 factors, such as IL-12 and CXCL9, was upregulated. This change in TAM phenotype was associated with increased antigen presentation, CTL infiltration and function, and tumour cell lysis. (63) Other agents have also revealed therapeutic potential by affecting macrophage polarity. For instance, bisphosphonates are administered to advanced breast and prostate cancer patients to reduce cancer-induced bone disease. They have been shown to act on macrophages, derived from the same progenitors as osteoclasts, by downregulating their production of angiogenic factors and MMP-9 and upregulating Inducible nitric oxide synthase (iNOS), which is important for macrophage cytotoxic activity. (64)

Another study in humans and mice evaluated the capacity of a CD40 agonist to activate macrophage tumoricidal activity. Treatment of pancreatic ductal adenocarcinoma patients with demcitabine chemotherapy and agonist CD40 antibody increased the response rate from 5.4% with demcitabine alone to 19% with the combination therapy. A 30% response rate was observed for the same treatment in mice, and the response was dependent on macrophages that became tumoricidal in response to CD40 activation. TAMs also upregulated their expression of IL-12, MHC class II, and costimulatory molecules necessary to present antigen to and activate tumour specific T cells. However, no significant increase in patient survival was observed. (65, 66)

One of the most striking anti-tumour responses observed in a mouse model triggered by macrophage repolarization was seen by Guiducci et al. (2005) in mice bearing mammary carcinomas or colon carcinomas. The mice were treated with a combination that showed a synergistic effect in promoting anti-tumour immunity: CpG ligand for TLR9-activating receptor on macrophages, plus anti-IL-10R and CCL16 macrophage chemokine. TAMs that had previously secreted IL-10 switched phenotype to TNF α and IL-12 producing macrophages, and increased their production of nitric oxide. 60% and 90% of the mice rejected colon and mammary tumours, respectively. About 30% of the response depended on macrophage tumoricidal activity, and the rest of the response depended on T cell

cytotoxicity. Tumour specific CTL activity was observed as early as seven days after treatment. (67)

However, there are potential caveats to skewing macrophage phenotype, or inhibiting their recruitment. M2 macrophages play an important role in resolving inflammation via their immunosuppressive characteristics and their mediation of tissue repair at sites of injury or infection. (2) Promoting the cytotoxic activity of M1 macrophages at the expense of M2 macrophage functions may lead to persistent tissue damage without resolution or repair at non-tumour sites. M2 macrophages are essential for wound healing and restored tissue homeostasis. Without functional M2 macrophages, persistent tissue damage may lead to chronic inflammation, altered tissue homeostatic set points, and chronic disease. (68) For instance, suppression of M2 macrophage function has been shown to produce massive inflammation of the gut due to the role of these cells in controlling such inflammation. (68) Also, immunosuppressive macrophages are required to reduce reactivity against apoptotic cells in the spleen. Therefore, inhibition of M2 macrophage function may promote responses against self molecules such as DNA, a response found in systemic lupus erythematosus and related autoimmune syndromes. (68) At the site of the tumour, persistent M1 macrophage activity and the resulting tissue damage may further drive chronic inflammation and the immunosuppressive activity of cell types other than macrophages. Indeed, other immune cells follow the same general polarity seen in macrophages with respect to their role in tumour progression. For instance, neutrophils have been shown to mirror M1-M2 macrophage polarity and can be classified into N1 and N2 neutrophils. N1 neutrophils are tumour cytotoxic and stimulate antigen presentation by dendritic cells as well as the tumour cytotoxic activity of natural killer cells. N2 neutrophils are induced by TGF- β and are immunosuppressive in addition to contributing to tumour cell proliferation, angiogenesis, and invasion. (69) Therefore, it is uncertain whether therapies stimulating M1 function at the expense of M2 are effective and whether the potential benefits outweigh the risks of inflammatory disorders. Additionally, it is not clear how long stimulated M1 activity can last without triggering a wave of immunosuppression and pro-tumorigenic activity from other immune cells recruited to the tumour site.

Conclusion

Immune cells have opposing properties in tumour progression, and the manipulation of their effector functions holds therapeutic potential. Adoptive T cell transfer and CTLA-4 or PD1/PDL1 inhibition have so far resulted in strikingly durable responses in a small but significant proportion of patients. There is evidence for the synergistic effect of coupling immune-based therapies with other cancer therapies, from chemotherapy and irradiation to oncogene inhibitors. Finally, skewing the phenotype of macrophages to suppress their tumour-promoting M2 functions and enhance their M1 tumoricidal activity and antigen presentation may be a significant component of cancer immunotherapy, since macrophages are the most abundant immune cell in the tumour stroma and have important contributions to tumour growth and invasiveness. The extensive crosstalk between macrophages and T helper cells further supports the promise of skewing macrophage polarity in order to mount effective tumour cytotoxic responses and inhibit pro-tumorigenic and immunosuppressive macrophage-T cell interactions. Therefore, future developments in cancer immunotherapy should strive to regulate these opposing networks of immune cells and their interactions in order to enhance the cooperative activity of Th1 cells, M1 macrophages, and CTLs while suppressing M2 macrophages, Treg cells, and other inhibitory interactions that reduce the effectiveness of tumoricidal responses. However, systemically tipping the balance between regulatory immune cells and pro-inflammatory immune cells may come at the cost of inflammatory disorders, resulting in autoimmunity and chronic disease. Developing cancer immunotherapies will benefit from efforts to target these therapies specifically to the tumour microenvironment, as well as from targeting potential responders.

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Keywords

Lewy bodies: protein aggregates present in neurons afflicted by neurodegenerative diseases

Dementia with Lewy Bodies: a progressive dementia closely resembling Parkinson's disease and Alzheimer's disease; characterized by protein aggregates diffused throughout the brain

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Dementia With Lewy Bodies: An Overview

Abstract

Background: Dementia is a neurocognitive disorder that involves multiple cognitive deficits, including memory impairment. Dementia occurs in a variety of disease processes, including Alzheimer disease (AD) and dementia with Lewy bodies, the two most prevalent neurocognitive diseases. This paper reviews the signs and symptoms, neuropathology, diagnosis, prognosis, and treatment of Dementia with Lewy bodies (DLB).

Methods: Terms searched included "Lewy body dementia," "Lewy body disease," "cognitive disorders," and "neurodegenerative diseases." Priority was given to peer-reviewed sources published within the last five years.

Summary: In addition to standard neurocognitive disorder symptoms, patients with DLB present clinically fluctuating cognition, visual hallucinations, and Parkinsonism as well as a variety of other symptoms with lower diagnostic sensitivity. Clinical signs, cognitive assessments, and radiologic imaging are used to diagnose DLB as being distinct from disorders like AD, Parkinson disease dementia (PDD), delirium, and normal aging changes. Interventions for this disease may be pharmacological or non-pharmacological. Pharmacological treatments include cholinesterase inhibitors, Levodopa, and selective serotonin reuptake inhibitors or serotonin-norepinephrine reuptake inhibitors. Non-pharmacological interventions include occupational therapy, cognitive stimulation, and physical activity.

Introduction

Dementia is a devastating diagnosis that significantly burdens those of advanced age. Its incidence is increasing in correlation with the world's increasing mean age; the 2050 projected morbidity for dementia is 60-114 million individuals worldwide. (2) Dementia impacts patients' social and occupational functioning, reasoning, memory, capacity for new learning, self-perception, and interpersonal interactions. (3, 4)

Dementia with Lewy bodies (DLB) is the second most prevalent type of dementia. (2, 5, 6) In addition to dementia, individuals with DLB have a varied symptomatology that ranges from visual hallucinations to Parkinsonism, and from sleep complications to alternating cognizance. (5,7) DLB has a unique clinical picture and treatment regimen, so proper diagnosis and management are integral to patients' well being.

Signs and Symptoms

DLB is progressive in course and insidious in onset. (7-11) Like other types of dementia, it is characterized by memory impairment, significant occupational or social decline, and apraxia, agnosia, and disturbances in executive functioning. (3, 12) The diagnostic characteristics of DLB are defined by the consensus criteria, a set of evidence-based guidelines determined by a panel of medical experts. (7)

Central, core, and suggestive features according to the consensus criteria are used to clinically establish a diagnosis of probable or possible DLB (see Table 1). The central feature, which must be present for any DLB diagnosis, describes patients with cognitive decline that interferes with normal social or occupational activities; deficiencies in attention, execu-

tive function, and visuospatial abilities; and memory impairment evident upon progression. (7, 8) Core features are specific characteristics that help identify probable or possible DLB. Suggestive features are diagnostically significant signs and symptoms that occur commonly but with lower specificity than the core features. A probable diagnosis of DLB can be made if two or more core features are present or if one core feature and one or more suggestive features are present. A possible diagnosis of DLB can be made if one core feature is present or if one or more suggestive features are present. (7,8)

Patients may also present with supportive features, which lack diagnostic specificity. Table 1 catalogs an inclusive list of diagnostically significant signs and symptoms.

Core Features

The core features of DLB are fluctuating cognition, visual hallucinations, and features of Parkinsonism. Fluctuating cognition involves changes in attention and alertness, daytime lethargy, more than two hours of daytime sleep, staring into space, and disorganized speech. (7-9, 13) Fluctuations from coherence to confusion can occur in the span of a moment, or across several weeks. (13)

Visual hallucinations may be the most useful symptom in diagnosing DLB because they rarely occur in other types of dementia. (7, 13) Hallucinations are often visual, intricate, and recurrent, but may less frequently manifest in the auditory, tactile, gustatory, and olfactory modalities. (5, 7, 9, 13, 14) Patients with DLB often report complex, "lively and colourful," hallucinations in "scenic sequences". (1)

Parkinsonism, or symptoms of Parkinson's disease, may occur in 60-92% of DLB patients. (5) Parkinsonism presents as spontaneous extra-

Table 1. Clinical diagnostic criteria for DLB^a

Central feature (must be present for a diagnosis of DLB)*:

Progressive cognitive decline
Impaired social or occupational function
Memory impairment often evident with disease progression
Deficits in attention, executive function, and visuospatial abilities

Core features:

Varied attention and alertness in fluctuating cognition
Detailed, recurrent visual hallucinations
Parkinsonism

Suggestive features:

REM sleep behaviour disorder
Severe antipsychotic sensitivity
Low dopamine transporter uptake in basal ganglia

Supportive features:

Syncope and repeated falls
Loss of consciousness
Severe autonomic dysfunction
Hallucinations in other modalities
Systematized delusions
Depression
Certain radiologic signs

Table 1: Table adapted from "Diagnosis and management of dementia with Lewy bodies: third report of the DLB consortium"⁷ Abbreviation: DLB, Dementia with Lewy Bodies. *One central feature or one or more core features indicates possible dementia. Two or more core features or one core feature and one or more suggestive features indicate probable dementia.

pyramidal symptoms, abnormal motor symptoms thought to result from a dopaminergic deficiency. (5, 7, 9) Patients often move rigidly, take small, slow, shuffling steps, and have a blank, emotionless expression. (5) Patients also tend to have a stooped posture, gait changes, and facial immobility. (7, 9, 13) Cogwheeling (ratcheting muscle tremors), bradykinesia (moving in slow motion), and fine-movement apraxia (inability to plan and execute movements) may also signify Parkinsonism in DLB. (9, 13)

Suggestive Features

The three suggestive features of DLB are REM sleep behavior disorder (RBD), severe sensitivity to typical antipsychotic medications, and low dopamine transporter uptake in the basal ganglia. (5, 7)

In RBD, individuals act out their dreams by moving around or vocalizing. (5, 7, 13) Bed partners can provide the history, or RBD can be confirmed by polysomnography. (7) RBD can be present years before other symptoms of DLB manifest. (5, 7-9)

Fifty percent of DLB patients react strongly to typical antipsychotics. (1, 7, 15, 16) Antipsychotics may cause the appearance of extrapyramidal symptoms (that can resemble Parkinsonism) for the first time, increase mortality, and exacerbate confusion and gait imbalance. (5, 7, 16) Neuroleptics, therefore, are contraindicated. (7)

Neuropathology

DLB is an alpha-synucleinopathy, an abnormal precipitation of alpha-synuclein proteins in the brain. (7, 10, 17, 18) DLB is characterized by the histological presence of Lewy bodies, or Lewy neurites. (2, 18) Lewy bodies are presynaptic aggregates of alpha-synuclein protein that occur in cortical and subcortical regions in DLB. (1, 7, 10, 11, 17, 18) It is currently thought that alpha-synuclein aggregates contribute to neurodegeneration, which results in a presynaptic neurotransmitter deficiency. (18) Acetylcholine

and dopamine paucity produce the overt clinical symptoms. (5, 18)

Cerebrospinal fluid (CSF) analyses have been suggested to link the presence of alpha-synuclein in the CSF and alpha-synucleinopathies like DLB, but the value of these tests is contentious because of false positives resulting from blood contamination during lumbar puncture and false negatives due to paradoxically lower-than-expected levels of alpha-synuclein in certain DLB patients. (7, 10)

Diagnosis

DLB can be challenging to diagnose due to inconsistency in symptomatic presentation and similarities in presentation to other diseases. (1, 5, 7, 10, 11, 19, 20) Although autopsy is the only conclusive way to confirm a diagnosis of DLB, patient history, physical examination, lab findings, cognitive assessments, and radiologic exams may help the clinician diagnose DLB. (5, 7)

Rating scales and cognitive assessments

The Mini-Mental State Exam (MMSE) is the most frequently used cognitive functioning evaluation for dementia. (4) The MMSE tests attention, short-term memory, visuospatial functioning, language, and orientation. Cognitive impairment and suspected dementia are signified by a score of 25 or lower (out of a perfect score of 30). (4, 21) While useful for establishing the presence and severity of dementia in the patient, the MMSE has several inherent weaknesses. It does not differentiate different types of dementia. (9, 7, 21) In DLB, memory impairments may not be apparent until later stages of the disease, and therefore may not be initially identified by the MMSE. (5, 7) The MMSE is prone to error when patients start at a high baseline intelligence, have never attained an eighth-grade level education, or are not native English speakers. (4, 15)

Other assessments may help the clinician identify or quantify impairment. The Clock Drawing Test, where the patient is instructed to draw an analog clock on a blank page, is most advantageous as a screening test due to its ease and speed of administration. (4) The Montreal Cognitive Assessment is a screening assessment available in multiple languages and sensitive to initial cognitive changes in highly educated individuals. (4) The Saint Louis University Mental Status Examination is also sensitive to early neurocognitive impairment, and accounts for educational level (whether high or low) in its assessment. (21) The Instrumental Activities of Daily Living assesses an individual's ability to complete tasks that are fundamental to independent living and is often used for functional evaluation prior to admission to long-term care facilities. (21) The Blessed Dementia Scale provides an evaluation of cognitive and behavioral functioning through observation by a caregiver over the duration of 6 months. (21)

Neurological Imaging

New research on neurological scans of patients with DLB may be the future of diagnostic criteria. Functional MRI findings indicate significant connectivity differences between patients with DLB and patients with Alzheimer disease (AD). DLB patients tend to have increased connectivity in the putamen and the inferior parietal cortex but decreased connectivity in the medial prefrontal cortex and the primary visual cortex. (20) MRI also demonstrates hippocampal atrophy in AD patients and preserved hippocampus volume in DLB patients. (6) Grey matter atrophy on MRI in DLB patients is similar to that in AD patients and correlates with cognitive decline. (6) Single photon emission CT (SPECT) scans have advantageous sensitivity and specificity of differentiating DLB from AD and normal individuals compared to clinical diagnosis. A SPECT scan with a greater likelihood of DLB shows decreased semi-quantitative uptake in the posterior putamen. (19) PET scans yield amyloid- β (an abnormal protein that accumulates in AD) deposition in AD and in concomitant AD and DLB, but not in sole DLB. (6) Although these imaging studies may eventually support the clinician in more accurately identifying DLB, there is not presently enough research to establish these tests as a component of the diagnostic criteria. (7)

Differential Diagnosis

Sensitivity in differentially diagnosing dementia is low. (7, 11, 19) DLB must be carefully differentiated from other common types of dementia that present similarly. A diagnosis of DLB should be made over time to best assess its characteristic fluctuating presentation and the full range of manifested symptoms. (8) Accurate diagnosis of DLB has a significant impact on the patient's course of treatment.

Alzheimer Disease

AD and DLB can co-occur in as many as 35-90% of cases. (5, 12) Several characteristics differentiate the two discrete processes. Visual hallucinations occur less frequently in patients with AD as compared with DLB patients. (14) Memory impairment is not as apparent in early-stage DLB as in AD (5, 13). Neuropsychologically, frontal executive function is worse in DLB than AD (11, 13). DLB has more profound cholinergic defects than AD, and as a result, more significant improvements from Cholinesterase inhibitors (ChEIs) in LBD have been hypothesized. (2, 5, 6) Functional MRIs may help differentiate the two diseases. (5, 20)

Parkinson Disease Dementia

DLB and Parkinson disease dementia (PDD) have the same underlying pathology of Lewy body disease. (7, 11, 17) Like DLB, PDD is an α -synucleinopathy. (2, 10) The main distinction between the two is an arbitrarily chosen time difference. If dementia develops within well-established Parkinsonism, the patient is diagnosed with PDD. If symptoms of dementia occur one year or more before symptoms of Parkinsonism, the patient is diagnosed with Lewy body disease. (7, 8)

Slight differences have been recorded. Lewy bodies are primarily located in the cortex in DLB and in the basal ganglia in PDD. (1, 8) PDD and DLB have similar global cognitive patterns, but DLB patients may perform worse on tests of attention, verbal memory, and executive function. (11) Rest tremor is less common in DLB patients than PDD patients. (7)

Prognosis

A study of late-life wellbeing found that a cognitive infrastructure with intact executive control processes is crucial to a sense of purpose, relationship quality, and opportunities for growth. (22) Because DLB is marked by a course of progressive and irreversible neurocognitive decline, quality of life is severely diminished. (7, 9, 10) Survival is, on average, 5-7 years after the onset of symptoms. (5, 8)

Treatment

Pharmacological

Cholinesterase inhibitors (ChEIs) are the recommended pharmacological treatment for DLB. (1,2, 6, 7, 10) ChEIs increase acetylcholine (ACh) concentrations at the synapse vis-à-vis inhibition of the enzyme (acetylcholinesterase) that degrades it. (2) This mechanism of action is aimed at correcting the pathologic deficit of ascending cholinergic neurons in DLB, which normally provide a link to higher brain centers in healthy brains. (2, 6) Current treatment provides symptomatic relief through increasing ACh, rather than correcting the underlying pathology of presynaptic α -synuclein aggregates. (1, 18) ChEIs have been shown to improve cognitive and neuropsychiatric symptoms in DLB patients. (5, 7)

There are three commonly used ChEIs: donepezil, rivastigmine, and galantamine. (2, 13) Greater efficacy of one particular ChEI has not yet been conclusively determined, although individuals may respond more favorably to a particular agent than another for several reasons. (2, 5) Donepezil and galantamine are metabolized by the liver, and rivastigmine is metabolized in the serum and excreted by the kidneys. Central action

of these medications causes their therapeutic effect, whereas peripheral action at cholinergic receptors is responsible for the dose-dependent side effects. (2) ChEIs may cause adverse effects including nausea, vomiting, anorexia, diarrhea, headache, dizziness, and syncope, which may be severe enough to limit their use. (1, 2) Polymorphisms and epigenetics play a role in the individual's response to therapy, although these factors are too multifarious to guide regimen selection at present. (2)

Other medications may be considered in the treatment of DLB. Levodopa, a dopaminergic agent often used in PD treatment, can be used to mitigate the motor symptoms of Parkinsonism that result from the loss of dopaminergic neurons of both pathologies. (5, 7, 13, 18) Selective serotonin reuptake inhibitors and serotonin-norepinephrine reuptake inhibitors are preferable if antidepressants are necessary because they do not have the anticholinergic side effects associated with other classes of antidepressants and because other classes may worsen hallucinations and cognitive symptoms in DLB patients. (5, 7) Anticholinergics and neuroleptics are contraindicated. (1, 7) Anticholinergics decrease the concentration of presynaptic acetylcholine, which exacerbates the underlying pathophysiology of DLB. DLB patients are more sensitive to the side effects of neuroleptics than the general population, and therefore more likely to experience adverse events (see "Suggestive Features"). (1)

Non-Pharmacological

Non-pharmacological interventions include motor and cognitive stimulation and training in activities of daily living (ADLs), namely, eating, bathing, dressing, using the bathroom, and perambulating. (21) Interventions have been shown to produce improvements in social behavior, ADLs, and night restlessness in patients with mild and moderate dementia. (23) Physical activity decelerates the progression of dementia, even in frail patients. (24) Individualized occupational performance treatments can improve task performance despite progressive cognitive losses. (12) Individuals with dementia may be able to benefit from education about their condition and may be capable of learning despite the degenerative nature of the disease. (12, 15) Further, systematized research of non-drug interventions should be conducted to establish a holistic treatment plan.

Conclusion

DLB is a complex condition to care for clinically. It must first be recognized as dementia, as signified by the individual's progressive cognitive decline, diminishing memory, and impaired social or occupational function. Screening tests like the MMSE or the Clock Drawing Test may aid the clinician in rapidly screening patients. Other cognitive evaluations are more suited for a thorough evaluation of the patient's mental and functional status. Further diagnostic imaging and studies may in the future come to occupy a niche in diagnosing DLB. The next obstacle is differentiating DLB from other neurodegenerative pathologies, such as AD or PDD, and identifying any comorbid conditions, especially when there exists a significant overlap of symptoms. A final stepping stone is selecting an appropriate treatment regimen. This can be done by considering the unique characteristics of the ChEI agents; selecting appropriate adjunctive therapy to manage comorbid symptoms such as Parkinsonism and depression; avoiding contraindicated classes of medications (anticholinergics and neuroleptics); and ultimately adjusting or fine-tuning the medication regimen based on the patient's response. Meticulous attention should be paid to the diagnosis and management of these patients in order to optimize quality and expectancy of life.

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Review Article

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Keywords

Alzheimer's disease: a progressive neurodegenerative disorder characterised by memory loss and associated with advanced age

Mindfulness-based interventions (MBI): a weekly set of group therapy, with prescribed cognitive exercises, based on Buddhist practices

Peripheral blood mononuclear cells: blood cells with a round nucleus; encompasses lymphocytes and macrophages among other immune cells

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The Promise of Mindfulness-Based Interventions as Therapies to Prevent Cognitive Decline

Abstract

Background: Alzheimer's disease (AD) is a neurodegenerative disorder that affects 5 million United States citizens. Many authors have proposed that mindfulness-based interventions (MBIs) have the potential to effectively prevent AD-associated pathology and symptomology. However, the fact that both meditation and AD are complex processes that involve a great number of biological pathways has made these phenomena particularly challenging to dissect. This review advocates the use of gene expression to investigate the mechanisms by which MBIs combat AD pathology.

Methods: Searches were performed using Thomson Reuters Web of Science. Ultimately, 85 journal articles were selected for their content as it pertains to the purpose of this review.

Summary: Peripheral blood mononuclear cells (PBMCs) may provide reliable measures of cerebral gene expression. Profiling their gene expression has demonstrated that MBIs may produce gene expression changes in many of the same pathways (inflammation, cellular stress, proliferation, synaptic function) and often in the opposite direction of disease-related deregulation. While AD is marked by shortened telomeres resulting in genetic turmoil, meditation has been documented to exhibit a positive effect on telomere maintenance. A comprehensive gene expression investigation is invaluable to reveal relevant molecular mechanisms and provide the foundation for exploring the interaction between MBI and AD.

Introduction

Alzheimer's disease (AD) is the sixth leading cause of death in the United States and affects more than 5 million citizens. By the year 2050, this number is expected to nearly triple to 13.8 million citizens. It is a particularly common disease in those of advanced age, with 11% of people age 65 and older and 32% of people 85 and older suffering from AD. Disease management carries heavy financial burden, with total costs of care expected to increase from a current annual \$203 billion to \$1.2 trillion by 2050. Unfortunately, these vast expenses do little for the patients, as AD cannot be treated, nor can its progression be slowed. The prevalence and associated expenses make AD uniquely damaging to society, but the neurodegenerative symptoms – of vastly unknown pathology – are the true detriment to quality of life. (1)

The promise of secondary prevention takes its roots in a 1988 postmortem examination of 137 nursing home residents that revealed 10 subjects whose cognition and functionality during life were unaffected despite mild AD pathology in the brain. Interestingly, these 10 subjects also had higher brain weights and greater neuron density, indicative of what is known as higher brain reserve. (2) Since these origins, the cognitive reserve hypothesis has evolved to encompass the fact that an individual with larger brain size and neuronal count has a reserve of brain matter that may protect the brain from neurodegenerative diseases by providing alternate physical pathways for effective neural signaling as other paths degenerate with disease. In fact, a meta-analysis of over 29,000 individuals showed that those with high cognitive reserve had a 46% reduced risk of developing dementia. (3) Further research has shown that increased cortical atrophy, higher levels of amyloid peptides in cerebrospinal fluid, and greater regional atrophy are necessary in order for individuals with higher cognitive reserve to express clinical symptoms of AD. (4)

At present, the main determinants used to predict cognitive reserve are education, occupation, and leisure time activities. (5) An activity aimed at building cognitive reserve that appears to be very promising is mindful meditation, or rather, the many varying forms of mindfulness-based intervention (MBI), which has been shown to invoke increases in volume in several regions of the brain and thickens the cerebral cortex. (6) Though far from being established therapies, MBIs are supported by a range of statistically significant data suggesting that they are effective in treating psychological disorders.

Purported Effects of MBIs on AD-Related Pathology

One brain region of interest on which MBIs have a neurotrophic effect is the hippocampus. In AD, the hippocampus atrophies even before subjects or their family members notice cognitive changes. (7) The decline in hippocampal volume is strongly correlated to AD and is especially linked to memory-loss symptoms. Remarkably, when two studies employed short, 8-week meditation periods, researchers found promising effects in the hippocampus. Hölzel demonstrated an increased left hippocampal volume in healthy subjects, while Wells described decreased hippocampal atrophy in patients with Mild Cognitive Impairment (MCI), a stage that is commonly considered to precede early AD. (8, 9) However, these particular studies had small sample sizes and were designed to detect structural differences, with less focus on the clinical diagnoses and cognitive changes that are relevant to AD improvement. Several other studies by Luders have demonstrated significantly larger right and left hippocampal volumes in long-term meditators as opposed

to normal controls. (10, 11) The importance of this recurring association is that meditation may build reserve and reduce atrophy – leading to less diminished cognition – in the area of the brain which is most damaged by AD. Therefore, it is very possible that meditation will at least prevent symptoms associated with decreased hippocampal function.

Meditation may also prevent AD symptomatology by other means. Although there is conflicting research amongst individual studies, meta-analyses and reviews suggest that depression is a risk factor for developing AD later in life, and one study reached the conclusion that MCI patients with depression have more than twice the risk of developing AD. (12-14) Depression occurs in approximately 20-30% of patients with AD, but it is unclear whether it is a risk factor for the disease, whether it is a symptom of neurodegeneration, or whether it is simply a reaction to cognitive decline. (15) No matter the case, MBIs are highly effective in decreasing the likelihood of depressive relapse. (16) Meditation has also been shown to counteract many other risk factors for AD, including high cholesterol, hypertension, stress, and cerebral hypoperfusion. (17-19)

Recent research has focused on the important role played by glucose in AD development, with a multitude of studies implicating defective glucose metabolism in patients with AD, plaque development, and decreased cognitive abilities. Several key findings are that higher blood glucose levels are a risk factor for AD; that AD patients have defective insulin signaling; and that insulin has been linked to neuroprotective factors that improve attention, memory, and cognition. More importantly, intranasal insulin therapy in patients with MCI and AD has produced cognitive improvements, possibly because the metabolism of the hippocampus is dependent on insulin for proper function. (20-22) At the same time, meditation upregulates insulin secretion, and as such, demonstrates the potential for decreasing cognitive decline. (23)

Ultimately, the aforementioned mechanisms are only a subset of the many ways that meditation can potentially mediate decreased cognitive decline and even improve cognitive function. For further reference, there are several recent review articles that further discuss the plethora of evidence to support this hypothesis. (24, 25) Of particular note is a review by Larouche et al. that identifies six main AD-related targets of MBIs: stress hormones, cytokines, serotonin, oxidation, white matter hyperintensities, insulin, and IGF-1. (26)

Research on MBIs in the context of AD is limited by few clinical studies and small sample sizes. However, the existing research does suggest that there is promise in the possibility that meditation can be an effective non-pharmacological intervention. Several MBIs have been linked to slowed age-related atrophy of the brain, although these studies did not address the effects of neurodegenerative diseases. (6) Studies on the effect of Kirtan Kirya meditation on neurodegenerative diseases have documented many improved cognitive parameters in patients with cognitive decline, MCI, and AD. (19) A study by Well and colleagues also examined an MBI (MBSR) as a potential preventative measure for dementia-related cognitive decline. The preliminary study demonstrated the potential for reduced hippocampal atrophy and improved functional connectivity in patients with MCI, yet it had too small a sample size for significance, was not structured to detect clinical differences in cognition, lacked a long-term follow up, and had an inadequate control group. (9)

The Power of Gene Expression Profiling

Less than twenty studies address overlap between MBIs and neurodegenerative diseases, and to date, none have addressed the topic with the complexity that is necessary to understand this potential interaction. While clinically relevant measurements of the effectiveness of MBIs on AD progression – such as fMRIs that measure cerebral size and tests that estimate various cognitive abilities – are unarguably pertinent in evaluating the relevant outcomes of MBIs, these measures do nothing to elucidate the mechanisms by which the two phenomena interact. Both meditation and AD involve physiological changes in an immense number of biochemical pathways, which complicates analyses of AD pathogenesis and MBIs. (27) With microarray profiling, it is possible to quantify the expression of many genes in parallel and identify differentially expressed

genes across diverging conditions. (28, 29) Thus, this technology may allow us to obtain a better understanding of convoluted molecular mechanisms.

In an effort to produce accurate gene expression profiles, it may be most relevant to investigate expression in cells of the CNS, and preferably those affected by AD pathology. Unfortunately, such analyses cannot be performed on the living, and post-mortem modifications of proteins and RNA degradation may perturb the resulting data. Still, there have been many studies investigating gene expression in human brains exhibiting a range of pathology, from MCI to severe AD. Such studies have reaffirmed the idea that AD is a systemic disease by finding significant differences in gene expression in a multitude of pathways. Those that were often down-regulated include general genomic transcription levels, transcription factors and associated signaling molecules, neurotrophic factors, signal transduction pathways, energy metabolism, synaptic vesicle pathways, calcium binding, and cytoskeletal formation. Those pathways that were consistently up-regulated were apoptotic signaling, pro-inflammatory signaling, cell adhesion, cell proliferation, protein synthesis, and lipid metabolism. (23, 30-37) However, many of these studies have had poor statistical power due mainly to small sample sizes. A recent study selectively reaffirmed decreased expression across a large number of genes involved in synaptic trafficking in MCI and AD patients as compared to healthy controls, with no significant difference between the two diseased states. (38) Further recent microarray studies have supported altered synaptic vesicle trafficking, as well as altered calcium signaling, cellular signaling, protein synthesis, metabolism, apoptosis, proliferation, transcription factor activity, inflammation, and neuronal proteins, among other differences that are concordant with the earlier gene expression analyses. (39-43) However, these were all post-mortem analyses of the brain and may contain gene expression data that is inconsistent with that of the living.

In 1996, it was noted that peripheral cells mimic many biological alterations that mark AD pathology. (44) Then in 2001, Tang and colleagues proposed the premise that distinct peripheral blood mononuclear cell (PBMC) expression patterns mirror differing neurological disease states, and used rat arrays with over 7000 genes to provide evidence for the hypothesized link. This team evaluated PBMC gene expression of rats that were subjected to ischemic strokes, hemorrhagic strokes, sham surgeries, kainite-induced seizures, and hypoxia or insulin-induced hypoglycemia, and concluded that these expression profiles are actually unique to each condition. Reporting gene expression changes in PBMCs after systemic hypoxia or hypoglycemia was not surprising since the cells are directly affected by the experimental conditions. However, changes in PBMC gene expression following non-systemic end-organ specific injury like brain ischemia, hemorrhage, or kainate-induced seizures was not self-explanatory, but confirmed the hypothesis that PBMC gene expression could be used as a fingerprint of cerebral neurological diseases. (45) Further study of PBMC gene expression patterns revealed consistency with distinct individual variations that were related to age, gender, the proportions of cell subtypes, and time of the day of sampling (46). Now, it is acknowledged that PBMCs can directly participate in the neurodegenerative processes and share many similarities with neurons in terms of biochemical machinery and AD-specific alterations. (47) Despite the fact that scientific research has yielded no validated peripheral markers for AD or any CNS-related events, PBMC gene expression analysis may be useful in understanding the multifactorial nature of neurological diseases, and especially those with systemic effects that extend past the central nervous system, such as AD.

There have been three studies that investigated the gene expression of PBMCs in AD patients. The first to address the issue was a study aimed to determine the gene expression profile of AD patients in comparison to age-matched controls in lymphocytes (a sub-category of PBMCs). RT-qPCR validation of microarray analyses excluded several genes – including those in the pathways of cellular and humoral immune responses and apoptosis – and determined that the $\alpha 2C$ -adrenoreceptor and defensin genes – genes involved in blood pressure and inflammation – were the only truly down-regulated genes. (48) Another study in 2007 also aimed to profile mononuclear cell gene expression in mild AD patients, using microarray analysis followed by RT-qPCR. They discovered similar

results to neuronal gene expression analyses, with up-regulation of genes involved in apoptosis, cell development, cell metabolism, CNS-synapse, inflammation, lipid metabolism, protein synthesis, as well as down-regulation in many genes involved in anti-apoptosis, cell development, cell metabolism, synaptic processes, cytoskeleton form and function, DNA repair, inflammation, lipid metabolism, mitochondrial function, cellular trafficking, signal transduction, and transcription and translation. In fact, 28% of up-regulated genes and 16% of down-regulated genes were previously reported to have similar expression in the post-mortem expression analyses, all 3 of which were discussed previously. Comparison to more studies could result in more overlap. However, no genes were found to be in common with the aforementioned 2005 lymphocyte study, although similar pathways were affected. (49) The many correlations between this study and previous post-mortem studies strengthen the case of PBMCs as a “window” into the CNS. The third study, like the first, used lymphocytes for microarray expression analysis. While this study had the smallest sample sizes, it did make a distinction in order to compare AD and MCI to controls. The authors also found differences in expression of several of the same genes or genes in the same family as those that were differentially expressed in the previous monocyte study, including genes involved in cellular signaling and metabolism. They also found a significant difference in expression of members of the ABC transporter family (with roles in membrane transport, translation, and DNA repair) between normal patients, MCI patients, and AD patients. (50) Overall, the three studies in PBMCs have suggested expression differences in many similar functional categories of genes as the studies in CNS tissue and especially in the categories of inflammation and intracellular signaling. (39) Non-microarray, targeted studies of PBMCs have revealed further differential regulation in MCI and AD patients that include changes in expression of genes in cholesterol metabolism, inflammation (and specifically, NF- κ B signaling), stress, proliferation, synaptic function, and iron homeostasis; the gene for transcription factor Sp1, which controls many AD-related proteins; and Scara1, an amyloid-beta receptor. (51-58)

Similar research has been conducted on the effects of MBIs on PMBC gene expression. The first study to do so analyzed practices that elicit the relaxation response (RR), which is particularly intriguing in that it does not delineate a specific form of meditation but rather allows for a variety of practices that induce similar physiological characteristics, mainly: decreased oxygen consumption, increased exhaled nitric oxide, and reduced physiological distress. (59, 60) This study compared the gene expression in long term practitioners (M) and 20 healthy controls (N1) who underwent eight weeks of RR training and were analyzed again (N2). In summary, the findings involved 1504 to 2209 differentially expressed genes in the 3 comparisons (N1 to N2, N1 to M, and N2 to M) with similar numbers of up-regulated and down-regulated genes. The differentially expressed genes were found to be involved in many recurring cellular pathways, including oxidative phosphorylation, ubiquitin-dependent protein catabolism, nuclear mRNA splicing, protein synthesis, cellular metabolism, NF- κ B signaling, and the regulation of apoptosis. (61) Another study that also analyzed PMBC gene expression did so in response to Sudarshan Kriya, a yogic and meditative practice. However, instead of using microarrays, the study employed qRT-PCR techniques specifically focused on genes involved in oxidative stress, DNA damage, cell cycle control, aging and apoptosis. The study concluded that there were significant differences in gene expression of practitioners of several proteins that protect the cell from oxidative damage, and increases in inflammation- and apoptosis-related COX-2 and stress response gene HSP-70. The results also present increasing trends in telomerase reverse transcriptase and BCL-2, which are related to cellular aging and anti-apoptosis, respectively. (62) It is of note to mention a third study that has some similar findings despite being performed on neutrophils (which are not PBMCs) and on the practice of Qigong. This study also found down-regulated genes in ubiquitin-dependent protein catabolism, cellular stress (with the exception of two up-regulated HSPs), and protein synthesis, as well as up-regulation of certain immunity-related genes. (63) From these experiments, it is quite clear that MBIs give rise to gene expression changes in many of the same pathways that are altered by AD. However, these studies all have their own downsides, including small sample sizes, unaccounted variables, and a lack of RT-qPCR validation. (64)

There are, however, several more recent studies that have provided

further insight into altered gene expression induced by meditative and yogic practices. A second study from the same RR researchers proceeded to further analyze the subject. Although focusing on temporal transcriptional changes associated with a single RR practice session after either long-term practice or an 8-week session, functional analysis revealed the potential impact of meditation on several pathways. The results demonstrated upregulated genes involved in telomere maintenance (which will be discussed in further detail below), calcium signaling, transcriptional regulation, insulin, and energy metabolism – which, as the authors propose, may aid in protection against aging and oxidation – and down-regulated genes in the apoptosis pathway, stress response pathway, and inflammatory pathways, including NF- κ B and associated molecules. (23) Another study that examined effects from only daily sessions of Sudarshan Kriya rather than longer-term practices reported significant rapid gene expression changes even from these single sessions. However, with several different approaches to gene ontology analysis, they were unable to identify any specific pathways that were affected. (65) Yet another study established decreased expression of pro-inflammatory genes and histone-deacetylases (HDACs, which generally repress transcription) based on custom pathway RT-qPCR of PMBC RNA of expert meditators after a day of intensive meditation. (66) Taken together, the two previously mentioned studies indicate that single daily sessions would most likely not be sufficient to combat AD pathology, and therefore serve to justify a longer intervention.

Only two studies have employed MBIs strictly as interventions – both lasting 8 weeks – and used PMBC gene expression as an outcome measure. One promising study demonstrated how 8 weeks of Kirtan Kriya Meditation reversed the pattern of up-regulated NF- κ B-related inflammatory gene expression and decreased immunity gene expression observable in the PBMCs of dementia caregivers. (67) Even more relevant is one study that employed an 8 week MBSR intervention and discovered decreased expression of pro-inflammatory genes (including NF- κ B) and correlated the reduction in inflammatory gene expression with a reduction in loneliness. (68) Despite being limited by small sample sizes, these studies demonstrate potential for meditation to combat at least some of the pathology of AD at the level of gene expression. There have been no published studies investigating the effects of MBIs on MCI or AD patients that have measured gene expression.

Telomeres

The effects of meditation and the pathology of AD converge on telomere maintenance. Telomeres are gene-poor, repetitive DNA regions that span 10-15 kb in humans. Active telomerase is crucial to maintain telomere length, and in turn, length is necessary to maintain cell viability; shortening beyond a critical length triggers a DNA damage response that results in cell cycle arrest or apoptosis. Telomere shortening is thought to contribute to the pathologies of degenerative diseases that often occur with age. (69, 70)

One of the first studies on AD and telomere length concluded that the PBMCs of AD patients had shorter telomeres than those of age-matched controls, and that T-cell telomere length specifically was correlated with Mini-Mental State Examination (MMSE) scores. The study also found an inversion correlation of T-cell telomere length with serum levels of the pro-inflammatory cytokine TNF α , suggestive of the concept that decreased telomere length is conducive of the immune dysfunction that is characteristic of AD pathology. (71) A study several years later provided supporting evidence, again citing shorter telomere lengths in T-cells of patients with AD-type dementia. (72) A third study demonstrated decreased peripheral blood leukocyte (PBL) telomere length in AD patients with a higher mortality correlated with shorter telomere length. (73) Another study also reported decreased PBL telomere length in AD, but comparatively longer hippocampal telomere length, probably due to gliosis, a glial cell response to cerebral injury that involves up-regulated telomerase. When comparing telomere length in the cerebellum – a region that is incapable of gliosis – to PBL telomere length, a correlation was found. (74, 75) Of particular note is a later study that reported significantly shorter

telomere length in monocytes of AD patients compared to controls. (76) An important implication of this research is that longer telomere length is associated with more normal functioning. Indeed, longer telomere length has been shown to be linked to improved cognitive function in healthy and cognitively impaired subjects, thereby making telomere length a target for therapeutic medicines and practices. (73, 77-78)

As mentioned above, increased telomerase expression after MBIs has been noted in microarray analyses. (23) However, this measurement of RNA transcription alone is insufficient evidence of increased telomerase function nor of increased telomere length. To pursue the question, there have been four randomized control trials that tested the effect of meditation on telomerase activity in PBMCs. The studies had varied controls, sample sizes (39-63), hours of practice (11-57), methodology for outcome measures, populations (overweight, chronic fatigue, long-term meditators, dementia caretakers), and meditative practices; yet all studies reported increased telomerase activity in the meditative group. (79-82) Together, these four studies display a significant combined effect size of 0.46. (83) However, an important point is that increased telomerase activity does not necessarily translate increased telomere length, as telomerase must access the DNA in order to extend it. One trial investigated the effect of meditation on actual telomere length, and though restricted by a small sample size, it demonstrated an increasing trend in relative telomere length (RTL) and a significantly longer RTL in women. (84) No randomized controlled trials have examined the effect of meditation on telomere length or telomerase activity in MCI or AD patients.

Concluding Remarks

It is necessary to first validate the yet-unverified principle that MBIs may prevent the progression of AD pathology before delving into intricate dissections of the relevant molecular interactions. Furthermore, the time period of intervention must be refined; is prevention best accomplished with lifelong commitment to an MBI, or can it be employed as a short therapy when the disease is already visible; and if so, at what stages will it be effective? These questions can best be addressed by large, highly controlled studies which measure clinically relevant symptoms of AD progression, such as cognitive decline and cortical atrophy. It is important to note that while gene expression may identify candidate interfaces of AD-MBI interaction, it only creates the foundation for functional experiments to clarify and explain the overlapping pathways. Furthermore, a necessary supplement to these investigations would be to isolate the epigenetic mechanisms that are at the root of the changes in gene expression.

AD and meditation are two complicated processes that involve changes in a great multitude of unique and overlapping pathways. As science continues to push forward the frontiers of high throughput information processing, the veiled particulars may emerge to provide a clearer understanding of their interaction. However, the genetic and molecular information of the present day stands in strong support of the hypothesis that meditation may be an effective means for the prevention of AD progression. The scientific world awaits a comprehensive gene expression study that can form the basis for a new age of effective, noninvasive dementia treatment.

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