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Full Length Research Paper

In vitro rat lymphocyte proliferation induced by *Ocinum basilicum, Persea americana, Plantago virginica*, and Rosa spp. extracts

R. Gomez-Flores[1]*, L.Verástegui-Rodríguez[1], R. Quintanilla-Licea [2], P. Tamez-Guerra[1], R. Tamez-Guerra[1] and C. Rodríguez-Padilla[1]

[1]Departamento de Microbiología e Inmunología, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, NL. México.
[2]Departamento de Química, Facultad de Ciencias Biológicas Universidad Autónoma de Nuevo León, San Nicolás de los Garza, NL. México.

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Medicinal plants have been used for centuries and have become part of complementary medicine worldwide because of their potential health benefits. Some of their metabolites have been successfully used directly in the treatment and prevention of infectious diseases and cancer, or indirectly by stimulating the immune system. In the present study, we investigated the effects of methanol and aqueous extracts of the Mexican plants *Ocinum basilicum*, *Persea americana, Plantago virginica*, and *Rosa spp.* on *in vitro* rat lymphocyte proliferation. Methanol extracts of *O. basilicum*, *P. americana, P. virginica*, and *Rosa spp.* stimulated up to 80, 16, 69 and 66% lymphoproliferation, respectively, whereas their respective aqueous extracts induced up to 83, 48, 31 and 83% lymphoproliferation, as compared with untreated controls. The effect of *O. basilicum* aqueous extract at concentrations of 31.25, 62.5, 125 and 250 μg/ml on lymphoproliferation was significantly different ($p < 0.05$) than the effects of *P. americana* and *P. virginica* at the same concentrations. We also observed that the lymphoproliferative effect of *Rosa spp.* aqueous extract was significantly higher ($p < 0.05$) than that of the methanol extract. Methanol and aqueous vehicles did not affect lymphocyte viability nor proliferation activity. The observed immunostimulatory effect may be of benefit in increasing the pool of lymphocytes in immunodeficiency patients.

Key words: *Ocimum basilicum*, *Persea americana, Plantago virginica, Rosa spp.,* immunoregulation, lymphocyte proliferation, phytomitogens, mexican plants.

INTRODUCTION

In the last three decades there has been a significant increase in the use of natural products in health care, and their potential applications in agriculture, pharmaceutical and food industry are being investigated. In fact about 30% of drugs used in industrialized countries are derived from plants (Kaufman et al., 1999). Plants play an essential role in the health care needs of native populations in America not only for the treatment of diseases, but also to improve the immunological res-ponse against many pathologies (Borchers et al., 2000). The use of preparations and infusions of plants to treat diseases has been practiced widely in Mexico and Central America for several centuries (Gomez-Flores et al., 2000; Kinghorn et al., 1998), but their effectiveness should be scientifically validated to increase the credibility of their use.

In the present study, we evaluated the effects of methanol and aqueous extracts of the widely distributed plants *Ocinum basilicum*, *Persea americana, Plantago virginica*, and *Rosa spp.* on *in vitro* rat lymphoproliferation, an activity that has not been previously studied or reported for these plants. *O. basilicum* L. (Lamiaceae)

*Corresponding author. E-mail: rgomez60@hotmail.com.

(basil, sweet basil) is mostly used in cosmetics, liqueurs, medicines, and perfumes. Its oil has antimicrobial and anti-inflammatory properties (Singh, 1999). Similarly, *P. americana* Mill (Lauraceae) (avocado) seeds are rich in tannins, and are used to treat diarrhoea; carotenoids and tocopherols from the fruit were shown to inhibit the *in vitro* growth of prostate cancer cell lines (Lu et al., 2005). Additionally, *P. americana* leaves have been reported to possess anti-inflammatory and analgesic activities (Adeyemi et al., 2002). *Plantago* species, on the other hand, have been reported to possess anti-inflammatory and antitumor properties (Samuelsen, 2000), and induce rat lymphocyte proliferation and nitric oxide and tumor necrosis factor-alpha production by rat peritoneal macrophages (*Plantago major*) (Gomez-Flores et al., 2000). Finally, people have used *Rosa spp.* (Rosaceae) (Rose wild) leaves for their laxative and astringent properties.

MATERIAL AND METHODS

Reagents and culture media

Penicillin-streptomycin solution, L-glutamine, and RPMI 1640 and AIM-V media were obtained from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS), sodium dodecyl sulfate (SDS), N,N-dimethylformamide (DMF), PBS, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Extraction buffer was prepared by dissolving 20% (wt/vol) SDS at 37°C in a solution of 50% each DMF and demineralized water, and the pH was adjusted to 4.7.

Animals

Sprague-Dawley male rats (200 – 220 g) were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). They were kept in a pathogen- and stress-free environment at 24° C, under a light-dark cycle (light phase, 06:00 - 18:00 h), and given water and food *ad libitum*. Animals were killed by asphyxiation in a 100% CO_2 chamber.

Collection and processing of plants

Plants were collected during summer in San Nicolás de los Garza N. L., México. *O. basilicum, P. virginica, P. americana* and *Rosa spp.* used in this study were identified by M.Sci. María del Consuelo González de la Rosa, Chief of the Herbarium of the Biological Sciences College at Autonomous University of Nuevo Leon, with voucher specimen numbers 024780 for *O. basilicum*, 024783 for *P. virginica,* 024782 for *P. americana*, and 024781 for *Rosa*. Leaves were rinsed, dried at 37° C for 36 h, and macerated in a coffee grinder (Proctor Silex E160B). Methanol extracts were prepared by placing 4.3 g of the leaf powder in 80 ml absolute methanol under shaking for 24 h at room temperature. The resulting extracts were centrifuged at 2800 rpm for 15 min, and supernatants were placed in 1 ml Eppendorf tubes, previously weighted. Then this material was dried under vacuum using a speed-vac concentrator (Savant Instruments Inc., Hicksville, NY), and dissolved in 300 µl methanol and 700µl RPMI 1640 medium supplemented with 10% FBS, 1% L-glutamine, and 0.5% penicillin-streptomycin solution (referred as complete RPMI medium). The aqueous extracts were prepared by placing 1g of the plant powder in 50 ml boiling distilled water for 10 min. The resulting extracts were then centrifuged at 2800 rpm for 15 min, and supernatants were placed in 1 ml glass tubes, previously weighted. Then this material was dried by lyophilizing, and dissolved in 300 µl methanol and 700 µl complete RPMI 1640 medium. Stock solutions of both methanol and aqueous extracts were then prepared at 0.5 mg/ml in complete RPMI 1640 medium, and sterilized by filtering through a 0.22 µ-membrane (Millipore, Bedford, MA).

Phytochemical analysis

The phytochemical analyses were done using standard procedures (Trease and Evans, 1996; Mojab et al., 2003; Olaleye, 2007).

Cell preparation and culture

Thymus was removed immediately after rat death (see above), and a single cell-suspension was prepared by disrupting the organ in RPMI 1640 medium as previously reported (Nowak et al., 1998). The cell suspension was washed three times in this medium, and suspended and adjusted to 1 X 10^7 cells/ml in complete RPMI 1640 medium.

T cell proliferation assay

T cell proliferation was determined by a colorimetric technique using MTT (Franco-Molina et al., 2003). Thymus cell suspensions (100 µl of 1 X 10^7 cells/ml) were added to flat-bottomed 96-well plates (Becton Dickinson) containing triplicate cultures (100 µl) of RPMI 1640 medium supplemented with 5% fetal bovine serum (unstimulated control), plant extracts at various concentrations, or vehicles (similarly processed as with plant methanol and aqueous extractions, but without plant material) for 48 h at 37°C in 95% air-5% CO_2 atmosphere. After incubation, MTT (0.5 mg/ml, final concentration) was added, and cultures were additionally incubated for 4 h. Cell cultures were then incubated for 16 h with extraction buffer (100 µl), and optical densities, resulting from dissolved formazan crystals, were then read in a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) at 540 nm (absorbance or optical density at 540 nm wavelength). The lymphocyte proliferation index (LPI) was calculated as follows:

$$LPI = \frac{A_{540}\ in\ plant\ extract\ treated\ cells}{A_{540}\ in\ untreated\ cells}$$

Percentages of proliferation, provided in the Results section, were calculated by using the lymphocyte proliferation indexes as compared with untreated controls (that is, an LPI of 1.6 indicates 60% increase in proliferation).

Statistical analysis

The results were expressed as mean ± SEM of the response of 3 replicate determinations from three independent experiments. Level of significance was assessed by Student's *t* test and ANOVA.

RESULTS

Phytochemical analysis

Phytochemical screening procedures were carried out to

Table 1. Phytochemical analysis of the Mexican plant methanolic and aqueous extracts.

Plant	Extract	Alk*	Flav	Sap	Terp	Tann	Sac
OB (basil)	Me	+	–	–	+	+	+
	Aq	–	–	–	–	–	+
PA (avocado)	Me	+	+	–	+	+	+
	Aq	+	+	–	+	+	+
PV (hoary plantain)	Me	+	–	+	+	+	+
	Aq	–	–	+	+	–	+
Rose spp.	Me	+/-	+	+/-	+	+	+
	Aq	–	+	+	–	+	+

*Alk =, alkaloids; Flav = flavonoids; Sap = saponins; Terp = terpenes; Tann = tannins; Sac = saccharides.

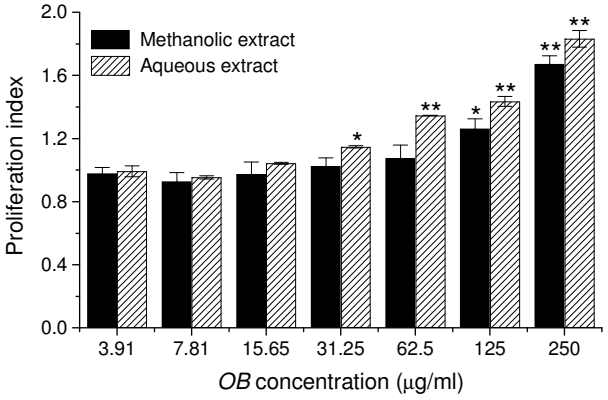

Figure 1. Lymphoproliferation induced by *Ocinum basilicum* methanol and aqueous extracts. Rat thymic cell suspensions were incubated in the presence or absence of various concentrations of *O. basilicum* methanol and aqueous extracts, after which lymphoproliferation was measured colorimetrically, as explained in the text. Data represent means ± SEM of triplicate determinations from three independent experiments. $^{**}p < 0.01$, $^{*}p < 0.05$ compared with *O. basilicum* extract-untreated control. Optical densities at 540 nm for untreated cells were 0.95 ± .01 and 1.1 ± .04 for methanol and aqueous extract treatments, respectively. A PI of 1 indicates no alteration on proliferation.

determine the potential active compounds that may be involved in the observed biological activities. Table 1 revealed that alkaloids were present in all methanol extracts. The aqueous extracts did not contain alkaloids. Furthermore, flavonoids were only detected in *P. americana and Rosa* methanol and aqueous extracts; saponins were detected in *P. virginica* and *Rosa* methanol and aqueous extracts; terpenes were present in all but aqueous *O. basilicum* and *Rosa* extracts; tannins were present in all but aqueous *O. basilicum* and *P. virginica* extracts; and saccharides were present in both methanol and aqueous extracts (Table 1).

Effect of plant extracts on T cell proliferation

O. basilicum methanol extract significantly ($p< 0.05$) stimulated 36 and 80% proliferation (from LPI data) of resident lymphocytes at 125 and 250 μg/ml respectively, whereas *O. basilicum* aqueous extract stimulated 15, 35, 44 and 83% proliferation of resident lymphocytes at 31.25, 62.5. 125 and 250 μg/ml respectively, as compared with untreated control (Figure 1); there was no difference between the effect of the aqueous and methanol extracts. The effect of *O. basilicum* aqueous extract at concentrations of 31.25, 62.5, 125 and 250 μg/ml on lymphoproliferation was significantly different ($p < 0.05$) than the effects of *P. americana* and *P. virginica* at the same concentrations. Optical densities at 540 nm for untreated control cells were 0.95 ± .01 and 1.1 ± .04 for methanol and aqueous extract treatments, respectively; these were used to calculate the LPIs.

In addition, *P. americana* methanol extract significantly ($p<0.05$) stimulated 17% proliferation (from LPI data) of resident lymphocytes at 250 μg/ml, whereas the aqueous extract stimulated 12, 20 and 48% lymphoproliferation at 62.5, 125 and 250 μg/ml respectively, as compared with untreated control; there was no difference between the effect of the aqueous and methanol extracts (Figure 2). The effect of *P. americana* aqueous extract at 250 μg/ml on lymphoproliferation was significantly different ($p < 0.05$) than the effects of *P. virginica* and *Rosa* at the same concentration. Optical densities at 540 nm for untreated cells were 0.73 ± .10 and 1.06 ± .04 for methanol and aqueous extract treatments, respectively; these were used to calculate the LPIs. Similarly, *P. virginica* methanol extract significantly ($p<0.05$) stimulated 29, 30 and 69% proliferation (from LPI data) of lymphocytes at 62.5, 125 and 250 μg/ml respectively, whereas the aqueous extract stimulated 11 and 31% lymphoproliferation at 125 and 250 μg/ml respectively, as compared with untreated control; there was no difference between the effect of the aqueous and methanol extracts (Figure 3). Optical densities at 540 nm for untreated cells were 0.84 ± .17 and 0.60 ± .08 for methanol and aqueous extract treatments, respectively; these were used to calculate the LPIs.

Furthermore, *Rosa* methanol extract significantly ($p < 0.01$) stimulated 66% lymphoproliferation (from LPI data)

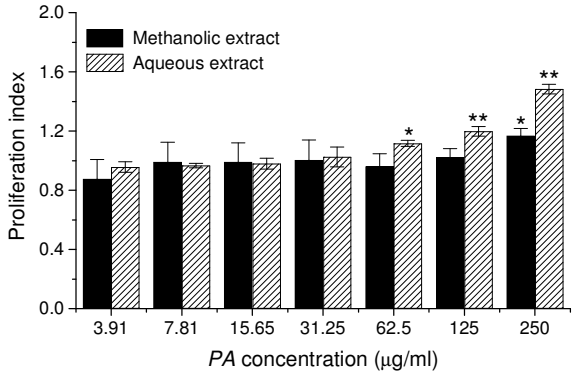

Figure 2. Lymphoproliferation induced by *Persea americana* methanol and aqueous extracts. Rat thymic cell suspensions were incubated in the presence or absence of various concentrations of *P. americana* methanol and aqueous extracts, after which lymphoproliferation was measured colorimetrically, as explained in the text. Data represent means ± SEM of triplicate determinations from three independent experiments. $^{**}p < 0.01$, $^{*}p < 0.05$ compared with *P. americana* extract-untreated control. Optical densities at 540 nm for untreated cells were 0.73 ± .10 and 1.06 ± .04 for methanol and aqueous extract treatments, respectively. A PI of 1 indicates no alteration on proliferation.

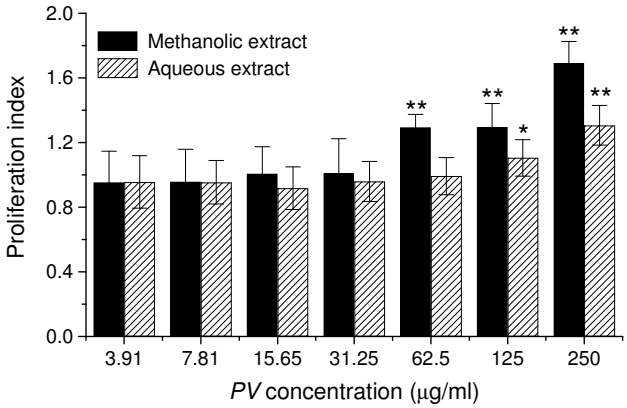

Figure. 3. Lymphoproliferation induced by *Plantago virginica* methanol and aqueous extracts. Rat thymic cell suspensions were incubated in the presence or absence of various concentrations of *P. virginica* methanol and aqueous extracts, after which lymphoproliferation was measured colorimetrically, as explained in the text. Data represent means ± SEM of triplicate determinations from three independent experiments. $^{**}p < 0.01$, $^{*}p < 0.05$ compared with *P. virginica* extract-untreated control. Optical densities at 540 nm for untreated cells were 0.84 ± .17 and 0.60 ± .08 for methanol and aqueous extract treatments, respectively. A PI of 1 indicates no alteration on proliferation.

at 250 μg/ml, whereas the aqueous extract stimulated 36 and 83% lymphocyte proliferative response at 125 and 250 μg/ml respectively, as compared with untreated control; the aqueous extract significantly ($p < 0.001$) induced higher lymphoproliferative activity than the methanol extract (Figure 4). Optical densities at

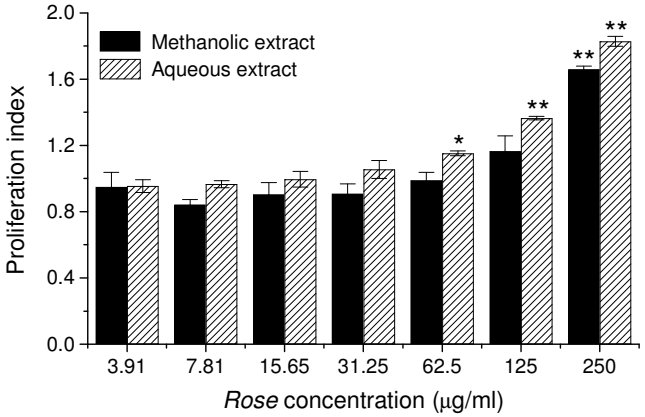

Figure 4. Lymphoproliferation induced by *Rosa* methanol and aqueous extracts. Rat thymic cell suspensions were incubated in the presence or absence of various concentrations of *Rosa* methanol and aqueous extracts, after which lymphoproliferation was measured colorimetrically, as explained in the text. Data represent means ± SEM of triplicate determinations from three independent experiments. $^{**}p < 0.01$, $^{*}p < 0.05$ compared with *Rosa* extract-untreated control. Optical densities at 540 nm for untreated cells were 0.92 ± .06 and 0.75 ± .02 for methanol and aqueous extract treatments, respectively. A PI of 1 indicates no alteration on proliferation.

540 nm for untreated cells were 0.92 ± .06 and 0.75 ± .02 for methanol and aqueous extract treatments, respectively; these were used to calculate the LPIs.

Vehicles for the methanol and aqueous extracts did not alter lymphocyte proliferation (Figure 5).

DISCUSSION

Natural products play an important role in the field of new drugs research and development (Jin-Ming et al., 2003). Knowledge of plant biologically active compounds and their mechanism (s) of action are desirable, not only for the discovery of novel therapeutic agents that would validate folkloric remedies, but also for the design of new active molecules or modification of current drugs against diverse maladies. One important application area is the immunotherapy; plant products have been shown to modulate the immune system (Spelman et al., 2006).

In the present study, we focused our study on some Mexican plants and demonstrated their potential to stimulate lymphocyte proliferation. Lymphocytes respond to antigen challenge by proliferating, expanding the antigen-specific lymphocyte clones and producing lymphokines, thus amplifying immune responses (Mackay, 1993). This effect can be mimicked by the use of polyclonal phytomitogens such as concanavalin A and phytohemagglutinin, which bind to certain sugar residues on T cell surface glycoproteins, including the T cell receptor and CD3 protein, and stimulate T cell proliferative response (Gajewski et al., 1989).

We found that all the tested extracts stimulated lym-

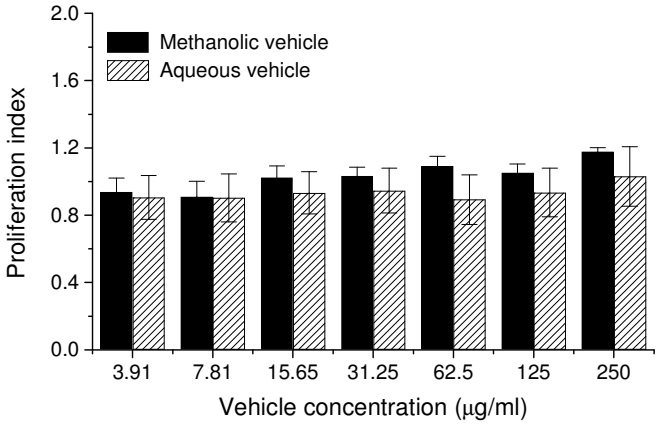

Figure 5. Effect of methanol and aqueous extract vehicles on lymphocyte proliferation. Rat thymic cell suspensions were incubated in the presence or absence of various concentrations of vehicles for the methanol and aqueous extracts (prepared as explained in the text), after which lymphoproliferation was measured colorimetrically. Data represent means ± SEM of triplicate determinations from three independent experiments. Optical densities at 540 nm for untreated cells were 0.59 ± .05 and 0.67 ± .09 for methanol and aqueous extract vehicle treatments, respectively.

lymphocyte proliferation. In the case of *O. basilicum,* such an activity was not due to the presence of alkaloids, flavonoids, terpenes, saponins, and tannins, because only saccharides were detected in these extracts (Table 1). However, it has been reported that *O. basilicum* leaves contain flavonoids and terpenoids (Grayer et al., 1996; Lemberkovics et al., 1998); in fact, a terpenoid called eugenol was shown to possess anti-inflammatory and antibiotic properties (Miele et al., 2001). In our study, *O. basilicum* extracts were found to induce the highest lymphoproliferative response (up to 80 and 83% for the methanol and aqueous extracts, respectively), as compared with untreated controls. Although, *O. basilicum´*s main reported activity is anti-inflammatory (Singh et al., 1999), its essential oil did not show significant effect on proliferation of murine lymphocytes stimulated with LPS and Con A as reported by others (Courreges and Benencia, 2002), but appeared to modulate both humoral and cell-mediated immune responses (Mediratta et al., 2002). In *P. americana* extract we detected the presence of flavonoids, terpenes, tannins, and saccharides (Table 1), as reported by others (Antia et al., 2005). Although, the aqueous extract of this plant was shown to possess anti-inflammatory and analgesic activities (Adeyemi et al., 2002), lectins from its seeds were not mitogenic for mouse lymphocytes (Meade et al., 1980), and "persin" from avocado leaves was shown to have antifungal properties and to be toxic to silkworms (Oelrichs et al., 1995), immune enhancing properties of this plant has not been previously reported. In addition, we observed the presence of alkaloids, saponins, terpenes, tannins, and saccharides in *P. virginica* extracts; the presence of such compounds has been reported in *Plantago* species (Samuelsen, 2000). The species *P. major* has been broadly used to treat diverse medical conditions ranging from pain to infectious diseases and cancer (Matev et al., 1982; Lithander, 1992; Grigorescu et al., 1973); particularly, anti-inflammatory properties were reported for this species (Herold et al., 2003), whereas Gomez-Flores et al. (2000) showed increased lymphoproliferative response of Con A-treated rat lymphocytes by *P. major* leaf extracts. Furthermore, Biringanine et al. (2005) reported *P. palmate*-mediated stimulation of nitric oxide and tumor necrosis factor-alpha production by activated macrophages.

Finally, the *Rosa* spp. aqueous extract was observed to significantly ($p<0.05$) induce higher lymphoproliferative activity than the methanol extract; this extract mainly contained flavonoids, saponins, and saccharides (Table 1). Although *Rosa* flowers were demonstrated to inhibit human immunodeficiency virus type-1 reverse transcriptase in-vitro (Fu et al., 2006), and Boyanova and Neshev (1999) showed inhibitory effect of rose oil products on *Helicobacter pylori* growth *in vitro*, immunomodulation by *Rosa* leaf extracts has not been previously reported.

Plant extracts are potentially curative. Some of these extracts can boost the humoral (Rehman et al., 1999) and cell-mediated immunity (Upadhyay et al., 1992) against viruses (Calixto et al., 1998), bacteria (Boyanova and Neshev, 1999), fungi (Ali et al., 1999), protozoa (Sharma et al., 1998), and cancer (Wong et al., 1994). In the present study, we demonstrated that various Mexican plant extracts stimulated murine lymphoproliferation. However, further studies are necessary to isolate the responsible active compound (s) and elucidate its mechanism of action. The regulation of immune parameters induced by plant extracts may be clinically relevant in numerous disease processes including chronic viral infections, tuberculosis, AIDS, and cancer.

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