ANALYSIS OF THE ANTIGEN- AND MITOGEN-INDUCED DIFFERENTIATION OF B LYMPHOCYTES FROM ASYMPTOMATIC HUMAN IMMUNODEFICIENCY VIRUS-SEROPOSITIVE MALE HOMOSEXUALS

Discrepancy between T Cell-Dependent and T Cell-Independent Activation

VERA J. P. TEEUWSEN,* TON LOGTENBERG,' KEES H. J. SIEBELINK,* JOEP M. LANGE,* JAAP GOUDSMIT,* FONS G. C. M. UYTDEHAAG,* and AB D. M. E. OSTERHAUS*

From the *Department of Immunobiology, National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands, 'Department of Clinical Immunology, Academical Hospital Utrecht, The Netherlands, and *Academical Medical Center, Amsterdam, The Netherlands

Five asymptomatic human immunodeficiency virus (HIV)-seropositive male homosexuals were immunized with the recall antigens tetanus toxoid (TT) and the three types of poliovirus present in diphtheria, tetanus, and polo vaccine. Four weeks after immunization, the in vivo response to booster immunization, the in vitro pokeweed mitogen (PWM)-induced IgG secretion, and the in vitro T cell-dependent and T cell-independent antigen-induced antibody response were assayed. Increase in serum antibody titer to TT and poliovirus was low and normal, respectively. In five subjects studied, a high rate of spontaneous IgG production, including antibodies directed toward HIV was observed. Addition of PWM to the cultures induced suppression of the spontaneous IgG secretion. Only one donor showed a slightly increased IgG production after stimulation with PWM. Peripheral blood mononuclear cells of four of the five HIV-seropositive individuals did not produce TT, or poliovirus-specific antibodies when stimulated with the respective T cell-dependent antigens. However, stimulation of these peripheral blood mononuclear cells with TT coupled to agarose beads, which was shown to be T cell-independent, resulted in the generation of IgG anti-TT antibody-forming cells.

Human immunodeficiency virus (HIV) is a recently discovered member of the Lentivirinae subfamily of the Retroviridae family, and is cytopathic and cytopathic for T lymphocytes with the cluster designation (CD) 4 (helper/inducer) phenotype (1-3). HIV has been implicated as the cause of acquired immunodeficiency syndrome (AIDS), of AIDS-related complex (ARC), and a degeneration of the central nervous system (1, 2, 4-7).

After an asymptomatic period of some weeks to several years, infection with HIV may result in disease symptoms caused by immunosuppression. The immune system of patients with AIDS or ARC is characterized by a decrease in CD4/CD8 (T helper/T suppressor) cell ratio, predominantly due to decreased numbers of circulating T lymphocytes with the CD4 phenotype (8). Besides the decrease in the number of CD4 lymphocytes, an intrinsic functional defect in the surviving T cell population is also responsible for the variety of immunologic abnormalities that have been described in this syndrome (9, 10). The most prominent abnormalities are a decreased blast transformation in response to mitogens and antigens, a decreased lymphokine production, a diminished expression of interleukin 2 receptors, a decreased alloreactivity, an impaired T cell clonability, the inability to provide help to B lymphocytes, a defective B lymphocyte function, and a defective monocyte function (9-21).

Since the introduction of serologic assays for the demonstration of antibodies to HIV, it has become apparent that the immunologic abnormalities, regularly observed in healthy male homosexuals during recent years, could be attributed to infection with HIV (22-24). However, little is known about the nature of the malfunction of the immune system in these asymptomatic HIV-seropositive individuals. In the present study we have investigated antigen-specific antibody production in vivo and in vitro of peripheral blood mononuclear cells (PBMC) isolated from asymptomatic HIV-seropositive male homosexuals who had recently been boosted with the antigens tetanus toxoid (TT) and the three types of poliovirus. In vitro antigen-specific T and B cell functions were studied by stimulating PBMC with pokeweed mitogen (PWM) or with soluble (T cell-dependent) and insolubilized (T cell-independent) antigens. Results of our study demonstrate that asymptomatic HIV-seropositive male homosexuals, as compared with age-matched heterosexual control individuals, have a subnormal increase in serum antibody titer after booster immunization, a severely impaired in vitro antigen-specific T cell function and/or accessory cell function, but an antigen-specific B cell function which appears normal.

MATERIALS AND METHODS

Subjects. Five 25- to 35-yr-old male homosexuals seropositive for HIV in several enzyme-linked immunosorbent assay (ELISA) systems
including Vironostika (see below), who had been diagnosed as clinically healthy, volunteered in this study. All of the men had been HIV-seropositive for at least 2 yr and so far exhibited a normal or slightly decreased CD4 count. Each was negative for anti-HIV-specific male homologous, had more than 0.7 x 10⁶ CD4 cells/ml. The observed decreased CD4/CD8 ratios were always the result of an increased CD8+ cell population. All of the HIV-seropositive volunteers remained clinically healthy during this study. Age-matched HIV-seronegative heterosexual controls, as well as normal healthy donors with HIV-seronegative female homologous, were immunized with diphtheria, tetanus toxoid, and polio vaccine during childhood and they had not been reboostered during at least the last 2 yr.

In the sampling of all subjects, unless otherwise stated, received an intramuscular booster immunization of one dose of diphtheria (D), tetanus (T), and polio vaccine routinely produced in the National Institute of Public Health, The Netherlands. The vaccine contained: 2.5 hflucoculat units (LF) D toxoid, 5 LF T toxoid (TT), and three poliovirus type 1 (Mahoney) 20 antigen units (DU), type 2 (MEF) 2 (DU), and type 3 (Saukett) 3.5 (DU) per dose. Blood samples were collected before and 4 wk after immunization. PBMC were isolated from heparinized blood by density gradient centrifugation on Ficoll-Isoaque at 1000 x G for 20 min and then were washed twice with RPMI 1640 (GIBCO, Grand Island, NY) supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml). PBMC were depleted of T cells by two cycles of rosette formation and separation on Ficoll-Isoaque with 2-aminoethylisothiourea bromide (Sigma, St. Louis, MO) as previously described (1). Non-rosetting cells were treated with RIV9, a CD3 monoclonal antibody (Dr. G. Kreeftenberg, National Institute of Public Health and Environmental Hygiene, Bihlthoven, The Netherlands) plus complement (Cedarlane Lab., Inc., Hornby, Ont., Canada). PBMC were depleted of monocytes by differential adherence to plastic as described elsewhere (26). These enriched B cell fractions contained less than 0.1% T cells as determined by immunofluorescence microscopy using a fluoresceinated CD2 reagent (Leu-5; Becton Dickinson, Sunnyvale, CA).

**Culture conditions.** For antigen-induced B cell differentiation, PBMC were cultured at 2 x 10⁵ cells/well and for PWM-induced B cell differentiation, PBMC were cultured at 10⁵ cells/well. Cultures were performed in 24-well, flat-bottomed plates (Falcon 3074; Becton Dickinson, Oxnard, CA) in RPMI 1640 containing penicillin (100 IU/ml), streptomycin (2 μg/ml), and 2 x 10⁻⁵ M β-mercaptoethanol (complete RPMI). For insolubilized TT (see below) induced B cell differentiation PBMC or purified B cells were cultured at 4 x 10⁴ cells/well in 96-well round-bottomed microtiter plates (Greiner No. 655180) were coated with 100 μl/well of 0.02% glutaraldehyde in phosphate buffer (pH 8.5) for 1 hr at 37°C. After washing with phosphate-buffered saline (PBS), wells were filled with 100 μl of TT (4 LF/ml) in phosphate buffer, pH 9.5. After 2 hr incubation at 37°C, plates were washed with PBS and were incubated for 45 min at 37°C with 1% bovine serum albumin (BSA) (Boehringer-Mannheim. Indianapolis). Plates were dried at room temperature and then incubated for 2 hr with TT and nonstimulated control PBMC were washed and added to TT-coated wells in 100 μl RPMI 1640. 1% BSA, at a cell density of 10⁵ cultured cells/ml. After 18 hr of incubation in a vibration-free incubator in a humidified atmosphere of 5% CO₂ in air at 37°C, wells were washed with PBS, 0.05% Tween-20, and incubated for 2 hr at 37°C with affinity-purified goat anti-human IgM conjugated to alkaline phosphatase (Tago Inc., Burlingame, CA) in PBS, 1% BSA, 0.05% Tween-20. After washing with PBS, 0.05% Tween-20, the substrate 5-bromo-4-chloro-3-indolylphosphate (5-BCIP) was added in gelling agarose. The substrate was a 2.3 mM 5-BCIP (Sigma Chemical Co., St. Louis, MO) solution in 0.1 M 2-mercaptoethyl-1-propanol buffer (Sigma) containing 5 mM MgCl₂. 0.1% Triton X-405 (Sigma), 0.01% NaN₃ and 0.6% 36°C gelling agarose (Sigma). After incubation at 37°C, IgG anti-TT-secreting cells became visible as blue spots which were counted after 18 hr using an inverted microscope.

**RESULTS**

In vivo response to booster immunization. To investigate the in vivo capacity to respond upon recall antigens, five asymptomatic HIV-seropositive male homosexuals were immunized with TT and the three types of poliovirus. Before and 4 wk after immunization, tetanus and poliovirus antibody serum titers were determined in an ELISA and in virus neutralization assay, respectively. As shown in Table I, all five donors had protective levels of tetanus antibodies (≥0.01 IU/ml) before immunization (31). Four of the five donors also had protective levels of poliovirus type 1.2, or 3 neutralizing antibodies (≥20) (32). After immunization with TT, the increase of anti-TT

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*Asymptomatic HIV-seropositive male homosexual.
**Pre- or post-booster vaccination.
†HIV-seronegative heterosexuals.
*≥9.
†n = 8.
serum titers proved significantly less than the increase observed in age-matched control individuals ($p < 0.01$, F-test). The differences in increase of virus-neutralizing serum antibody titers against poliovirus type 1, 2, or 3 between the asymptomatic HIV-seropositive donors, on the one hand, and, on the other hand, the age-matched controls were less evident (Table I). However, one seropositive donor (I) had an anti-poliovirus type 3 serum titer $\geq 2$ before and after immunization.

**In vitro mitogen-induced IgG secretion.** PBMC of the five asymptomatic HIV-seropositive male homosexuals, isolated 4 wk after booster immunization and PBMC of five nonimmunized healthy male heterosexuals were stimulated with PWM. After 10 days of culture, supernatants from unstimulated and PWM-stimulated cultures were assayed for the presence of total IgG and IgG anti-HIV. Unstimulated PBMC of the seropositive donors, but not of the heterosexual control individuals, spontaneously secreted IgG (Fig. 1A). Part of this spontaneously secreted IgG consisted of antibodies directed to HIV, as measured in ELISA (Fig. 1B). In PWM-stimulated cultures of PBMC from four out of five HIV-seropositive donors, total IgG secretion had decreased, whereas PBMC from only one seropositive donor showed a slightly increased IgG secretion as compared with the elevated IgG production by PWM-stimulated PBMC from the control individuals (Fig. 1A). Moreover, in PWM-stimulated cultures of PBMC from all five HIV-seropositive donors, HIV-specific antibody production had decreased (Fig. 1B). None of the control individuals produced HIV-specific antibodies after stimulation with PWM (Fig. 1B). In addition poliovirus neutralizing antibodies and antibodies directed to TT were not detected in supernatants of unstimulated or PWM-stimulated cultures of PBMC from HIV-seropositive donors.

**Antigen-induced T cell-dependent antibody production in vitro.** Four weeks after the booster immunization with TT and the three types of poliovirus, PBMC of the five asymptomatic HIV-seropositive male homosexuals and of five HIV-seropositive male heterosexuals were assayed for their capacity to produce specific antibody in vitro. To that extent PBMC were cultured with different concentrations of soluble TT, or poliovirus type 1, 2, or 3. PBMC from five HIV-seronegative control individuals produced anti-TT IgG (no anti-TT IgM, data not shown) and poliovirus type 1, 2, and 3 neutralizing antibody upon stimulation with the respective antigens (Figs. 2 and 3). This in vitro induced antibody response proved to be specific since, in cultures of PBMC, anti-poliovirus-producing cells were not detected after stimulation with TT: similarly after stimulation with poliovirus no production of anti-TT was observed (data not shown). After culturing of PBMC from the five HIV-seropositive donors with soluble TT, no TT-specific IgG or IgM (data not shown) production could be detected in a spot-ELISA (Fig. 2). After 10 days of culturing of PBMC from the HIV-seropositive donors with poliovirus type 1, 2, or 3, respectively, cells from four out of five donors failed to produce detectable levels of poliovirus-neutralizing antibody as measured in the virus neutralization assay. PBMC from the other seropositive donor did produce neutralizing antibodies against poliovirus type 2 and 3, but not against type 1 when stimulated with the respective antigens (Fig. 3).

**Mitogen- and antigen-induced lymphocyte proliferation.** Given the impaired T cell-dependent antigen or mitogen-induced B cell activation, proliferative responses upon stimulation with soluble TT (sTT) or PWM of PBMC from three of the HIV-seropositive individuals were evaluated. As shown in Table II, proliferative responses upon stimulation with PWM or TT were low compared with the responses of PBMC from HIV-seronegative controls, except for donor III who showed a response upon stimulation with PWM. This donor also demonstrated an increase in total IgG production after stimulation with PWM, as shown in Figure 1.

**Antigen-induced T cell-independent antibody production in vitro.** It has previously been shown that the
autoantigen thyroglobulin coupled to a solid phase can be used to induce antigen-specific secondary type B cell responses in vitro, independently of T cells (26, 33). In analogy, we coupled TT to Affi-Gel 10 (iTTr) to examine whether secondary anti-TT responses independently of T cells could be raised. To demonstrate the capacity of B cells to respond upon stimulation with iTT without the involvement of T cells and adherent cells, B cells of TT booster-immunized control individuals were purified as described in Materials and Methods.

PBMC and purified B cells were stimulated with serial dilutions of iTT and sTT and after 6 days of culture assayed for IgG anti-TT antibody-forming cells in a spot ELISA. Representative data from one control donor are shown in Table III. Stimulation of purified B cells and unseparated PBMC with iTT resulted in the production of IgG anti-TT. In contrast to PBMC, purified B cells did not respond upon stimulation with sTT. These data show that iTT activates human B cells independently of T cell help to the production of TT-specific antibody.

We used this method to analyze the T cell-independent responsiveness of B cells to TT from the HIV-seropositive donors. PBMC were stimulated with serial dilutions of iTT and examined for production of TT-specific antibody in comparison with production by PBMC from the HIV-seropositive controls. As shown in Figure 4, PBMC from both HIV-seropositive and HIV-seronegative men could be stimulated equally well to IgG TT-specific antibody production. No IgM TT-specific antibody-forming cells were detected (data not shown).

**DISCUSSION**

Previous studies on the function of the immune system in HIV-infected individuals have mainly been focused on patients with AIDS or ARC. These studies demonstrated a severely impaired antibody response induced by antigen or mitogen, without elucidating the underlying mechanisms (14–18). In the present report we have studied both T cell-dependent and T cell-independent activation of B cells from asymptomatic HIV-seropositive male homosexuals. To this end in vivo and in vitro antigen-specific immune responses were measured in TT and poliovirus (type 1, 2, and 3) booster-immunized asymptomatic HIV-seropositive male homosexuals and compared with those of similarly booster-immunized HIV-seronegative heterosexual male individuals.

Four weeks after immunization, all five seropositive donors showed a relative low and normal increase in serum antibody titers to TT and polioviruses respectively, suggesting B cell activation in vivo. Despite this in vivo observed responsiveness, T cell-dependent antigen- and mitogen-induced B cell differentiation in vitro proved to
be severely impaired in these individuals. PBMC from the asymptomatic HIV-seropositive individuals in contrast to those of control individuals failed to respond upon stimulation with the soluble protein antigen TT or the three types of poliovirus. For this impaired in vitro antigen-induced antibody synthesis, the following explanations may be considered: the absence of functionally active antigen-specific memory B cells, an insufficient T helper cell activity, or an impaired accessory cell function.

The absence of functionally active antigen-specific B cells could be excluded, since here we demonstrated that PBMC of both HIV-seropositive and -seronegative individuals could be triggered equally well to anti-TT IgG production by iTT, which has shown to stimulate human B cells, independently of T cell help and probably without the interference of adherent cells. The observed impaired antigen-induced T cell-dependent B cell responses, but normal T cell-independent B cell responses suggest that T cell function and/or accessory cell function rather than B cell function is disturbed in these asymptomatic HIV-seropositive male homosexuals. Consistently, proliferative responses of PBMC from the HIV-seropositive individuals upon stimulation with sTT proved relatively low when compared with those from HIV-seronegative control individuals. This finding confirms recently published data showing low antigen-induced responses of T cells from both patients with AIDS and asymptomatic HIV-seropositive individuals [9, 34].

The possibility that accessory cells rather than, or in addition to, T helper cells themselves, may have accounted for the observed impaired secondary immune responses cannot be ruled out from the data presented. Interestingly, it has been shown that both monocytes and macrophages are permissive for HIV infection and it is conceivable that a defective monocyte function as described in AIDS patients may also play a role in the unresponsiveness of asymptomatic HIV-seropositive individuals as reported here [35–37].

Patients with AIDS or ARC often show hyperimmunoglobulinemia with increased numbers of circulating immunoglobulin-secreting cells [14, 16, 17]. Here we showed that the PBMC of asymptomatic HIV-seropositive male homosexuals also spontaneously produced elevated levels of IgG, including antibodies directed toward HIV. Poliovirus-neutralizing antibodies or antibodies directed to TT were not detected in the culture supernatants of these PBMC isolated 4 wk after the booster immunization. Recently, Yarchoan et al. [18] demonstrated in a very sensitive precursor frequency determination that in patients with AIDS or ARC part of the spontaneously produced antibodies comprised antibodies toward HIV and to at least one recall antigen and frequencies observed for recall antigen-specific B cells were lower than those for HIV-specific B cells. The failure to detect spontaneously secreted antibodies directed to recall antigens may be inherent to culture conditions and sensitivity of the assays used, but is in agreement with the observation that lymphoblastoid B cells, spontaneously secreting antibodies to an immunizing agent, independent of T cell help, circulate only between 5 and 10 days after immunization [38, 39]. The detection of spontaneously produced IgG directed to HIV may reflect recent or continuous activation of HIV-specific B cells by the expression of HIV antigens in vivo. Furthermore, HIV has been
shown to be a T cell-dependent polyclonal stimulator in vitro, which may also have accounted for the spontaneous production of IgG, including antibodies directed to HIV (18, 40, 41). In four out of five seropositive donors, addition of PWM to the cultures resulted in a decreased spontaneous IgG production, whereas HIV-specific antibody production was decreased in all five donors tested. PWM-induced suppression of the spontaneous Ig secretion of in vivo activated B cells, as has been demonstrated in several systems, has been explained by the involvement of a subset of PWM-inducible, in vivo preactivated CD8+ suppressor T cells (42, 43). This may represent a nonspecific physiologic control system on activated B cell stages. More recently it was observed that in HIV-seropositive male homosexuals described in this report, the impaired in vivo and in vitro immune functions suppressive consequences of HIV infection, we conclude that the in vitro anergy to mitogens such as PWM not only appears in vitro, which may also have accounted for the spontaneous IgG production, whereas HIV-specific antibody response in human T and B cells using AET treated SRBC rosettes. J. Immunol. 127:1253.


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