Immunoregulation in Humans

CONTROL OF ANTITETANUS TOXOID ANTIBODY PRODUCTION AFTER BOOSTER IMMUNIZATION

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ABSTRACT Booster immunization of normal individuals with soluble tetanus toxoid resulted in the ability of the individuals' peripheral blood lymphocytes to synthesize immunoglobulin (Ig)G antitetanus toxoid antibody in vitro when stimulated by pokeweed mitogen. The capacity for this in vitro antitetanus toxoid antibody response developed within 14 days after booster immunization, reached a peak between days 36–50, and disappeared by day 60. The inability of pokeweed mitogen to stimulate antitetanus toxoid antibody synthesis in vitro before booster immunization was not due to excess suppression by thymus-derived (T) lymphocytes but reflected insufficient numbers of functionally specific helper T lymphocytes and bone marrow-derived (B) lymphocytes.

Antigen-specific T-lymphocyte suppression and decreased B-lymphocyte function were associated with the observed reduction of in vitro synthesis of antitetanus toxoid antibody from 20–60 days post-immunization. The in vitro kinetics of antitetanus toxoid antibody synthesis paralleled the synthesis of total IgG in that stimulation by pokeweed mitogen was required and that antibody secretion into the medium initiated by day 4 and increased through day 9.

INTRODUCTION

Circulating human peripheral blood lymphocytes (PBL)¹ can be simulated in vitro by pokeweed mitogen (PWM) to undergo a complex series of cellular interactions which culminate in the increased production of immunoglobulin (Ig)G, M, and A (1–5). This polyclonal stimulation of immunoglobulin involves bone marrow-derived (B) lymphocytes and several defined

populations of thymus-derived (T) lymphocytes whose functions include enhancement, suppression (1–5), and possible amplification (6) of the humoral immune functions. Identification of these T-cell subpopulations has resulted from differential sensitivity to irradiation and steroids (7–9), from expression of membrane receptors (10, 11), from exaggeration of certain functions during disease states (12–15), and from the fact the system is not constrained by allogeneic differences (2, 4, 5).

In humans, circulating lymphocytes represent cellular subsets different from those found in other lymphoid tissues as evidenced by differential sensitivity to antigens and mitogens (16, 17), and by the fact that circulating B lymphocytes poorly reexpress surface Ig after antibody stripping, whereas spleen and tonsil B lymphocytes regain surface Ig within 24 h (18).

The in vitro homeostatic role for circulating lymphocytes, and in particular those lymphocytes responsive to PWM, has not yet been carefully defined. Our approach for investigating the role of human circulating lymphocytes responsive to PWM is the quantitation of the functional abilities of specific regulating and antibody-producing cell populations in vitro after a perturbation of the immune system by in vivo immunization.

In this report we demonstrate that after booster immunization of normal individuals with tetanus toxoid, there is a selective increase in the capacity of the blood lymphocytes from the immunized individuals to produce antitetanus toxoid antibody in vitro after PWM stimulation.

METHODS

Purified tetanus toxoid was generously provided by Wyeth Laboratories (Marietta, Pa.). Rabbit anti-human IgG was obtained from N. L. Cappel Laboratories, Inc. (Cochranville, Pa.) and was purified by ammonium sulfate fractionation and DEAE (DE-52) chromatography. The specificity of the antiserum was confirmed by immunoprecipitation of radiolabeled

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¹*Abbreviations used in this paper:* BSA, bovine serum albumin; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; PWM, pokeweed mitogen.

myeloma proteins (19) and absorbed when necessary with the appropriate Ig molecules. Microtiter plates were obtained from Scientific Products (McGraw Park, Ill.) and bovine serum albumin, fraction 5, was obtained from Sigma Chemical Co. (St. Louis, Mo.).

Immunization. Normal individuals were given an intramuscular booster immunization with five flocculation units of soluble tetanus toxoid.

Lymphocyte preparation and separation procedures. Human PBL suspensions were prepared by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, N. J.) differential sedimentation (20) of heparinized blood obtained from normal volunteers between the ages of 25 and 35 yr. The blood was diluted with an equal volume of 0.15 M saline, and the suspension (30 ml) was layered over 15 ml of Ficoll-Hypaque (sp gr = 1.078–1.080). After centrifugation at room temperature (600 g, 35 min), the interface cells were removed and washed three times in minimal essential medium. T- and B-lymphocyte fractions were separated by density sedimentation of spontaneous rosettes formed by T lymphocytes and sheep erythrocytes. The procedure was modified in that the sheep erythrocytes had been pretreated with 2-aminoethylisothiuronium bromide (21).

Lymphocyte cultures. Either unfractionated, fractionated, or fractionated and recombined PBL were cultured in RPMI 1640 medium buffered with NaHCO_2 and supplemented with 1-glutamine (10 mM), gentamicin (0.05 mg/ml), and 15% heat-inactivated fetal calf serum. All cultures were done in a final volume of 1.5 ml in 13×100 -mm plastic tubes (Falcon 2027, Falcon Plastics, Div. BioQuest, Oxnard, Calif.) and contained PWM (Grand Island Biological Co., Grand Island, N. Y.) at a final dilution of 1/100 vol/vol. The tubes were incubated in a humidified atmosphere at 37°C with 5% CO_2 for 1–9 days.

Radioimmunoassays. The quantitative radioimmunoassay for antitetanus toxoid antibody and total IgG synthesized *in vitro* were performed in microtiter plates (22). For measurements of antitetanus toxoid antibody, the individual wells in microtiter plates were filled with tetanus toxoid at a concentration of 1 mg/ml and incubated overnight at room temperature. The following day, the tetanus toxoid was removed and each well individually washed with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). A 10% solution of BSA in PBS was added and after 1 h, each well was subsequently washed three times with 1% BSA in PBS. Samples of the culture supernates (maximum of 150 μl) were then added, the wells filled to capacity with 10% BSA in PBS, and the plates incubated 16 h at room temperature in a humidified chamber. The next day, the plates were washed three times with 1% BSA in PBS and each well filled with a solution containing 1 μg of DEAE-purified iodinated (^{125}I) (23) rabbit antihuman IgG (spec act 1,874–5,200 cpm/ng) in 10% BSA. After 6 h incubation at room temperature, the wells were washed three times with 1% BSA in PBS and eight times with tap water.

The binding of the antibody to the antigen-coated plates in the first incubation was complete by 20 h. The second incubation, in which an excess of iodinated antibody was used to detect the antitetanus antibody bound to the plate, was complete between 2–4 h. 1 U of antitetanus toxoid antibody was defined as equal to 1 ng of rabbit anti-human IgG bound to the plate.

The radioimmunoassay for total IgG synthesized in the cultures was similar to the antitetanus antibody radioimmunoassay with the exception that monospecific rabbit anti-human IgG (0.5 mg antibody/ml) was used for the initial coating of the plate. For each radioimmunoassay a 7-point standard curve of IgG ranging from 1–15 ng was performed in parallel with

the culture samples on each plate. The IgG values for the culture samples were calculated from this curve.

RESULTS

Radioimmunoassay for human IgG antitetanus toxoid antibody. The antitetanus toxoid antibody binding capacity of the tetanus toxoid-coated plates was determined by incubating triplicate wells with increasing quantities of human serum containing antitetanus toxoid antibody. Fig. 1 demonstrates that increasing quantities of serum added in the first incubation resulted in a subsequent increased binding of the second antibody with saturation being reached between 40,000–50,000 cpm. From the specific activity of the radioiodinated second antibody (1,874 cpm/ng), this was found to correspond to 20–25 ng/well of rabbit anti-human IgG. The binding of rabbit anti-human IgG to the plate was linear below 10 ng, and all experimental antibody levels were subsequently determined with diluted samples resulting in the binding of <12 ng/well of rabbit anti-human IgG.

The binding of serum antitetanus toxoid antibodies to the plate could be reduced by the inclusion of soluble tetanus toxoid in the first incubation (Fig. 2). Similarly, the binding of ^{125}I -rabbit anti-human IgG to the antitetanus toxoid antibody on the plate could be inhibited by the addition of nonradiolabeled human IgG (Fig. 2). 50% competition of the binding of the antitetanus toxoid antibody (equivalent to 12 ng rabbit anti-human IgG) to the plate occurred with 26 ng of soluble tetanus toxoid. 50% competition of the binding

FIGURE 1 Saturation of tetanus toxoid-coated plates with antibody. Increasing amounts of serum from an individual repeatedly immunized with soluble tetanus toxoid were added to triplicate wells in a microtiter plate previously treated 16 h with soluble tetanus toxoid (1 mg/ml). After 16 h incubation and subsequent washing, 1 μg DEAE-purified ^{125}I -rabbit (RAB) anti-human IgG (sp act = 1,874 cpm/ng) was added to each well. The bound radioactivity was determined after a 5-h incubation. The numbers in parentheses are the quantities of rabbit anti-human IgG bound as determined from the specific activity. Background value was 869 cpm.

FIGURE 2 Competition of the reaction by soluble tetanus toxoid and human IgG. The competition of antitetanus toxoid antibody binding to the plate by soluble tetanus toxoid in the first incubation was demonstrated by mixing 20 μ l of a 1:300 dilution of serum from an immunized individual (equivalent to 11.5 ng of a rabbit anti-human IgG bound) with increasing quantities of soluble tetanus toxoid (x). The competition of the iodinated rabbit anti-human IgG binding by soluble human IgG was demonstrated by the addition of increasing quantities of purified human IgG during the second incubation (o). The 100% binding value was 61,502 cpm. The specific activity of the iodinated antibody was 4,370 cpm/ng.

of the rabbit anti-human IgG to the antitetanus antibody on the plate was achieved with 112 ng of the IgG indicating that the second antibody was in excess. For each experimental group, a standard dilution of serum containing antitetanus toxoid antibody was added to control wells on each microtiter plate. Results from 30 individual measurements of this control sample, using 125 I-rabbit anti-human IgG with specific activities ranging from 1,874–5,230 cpm/ng, gave a value of 11.57 ± 2.2 U.

In vitro synthesis of antitetanus toxoid antibody and IgG after PWM stimulation. Unfractionated lymphocytes from recently boosted individuals (<60 days) synthesized up to 130 U antitetanus toxoid antibody in vitro over an 8-day incubation with PWM (Fig. 3). Secreted antitetanus toxoid antibody could be detected in the culture medium by day 4 and increased through at least day 9. No antitetanus toxoid antibody activity was detected on day 1 of culture nor in non-PWM-stimulated cultures precluding the possible carryover of serum antitetanus toxoid antibody on the lymphocytes. The synthesis of total IgG in the cultures showed similar kinetics to that of antitetanus toxoid IgG antibody with a somewhat higher quantity of total IgG appearing by day 4. All subsequent experiments were terminated and assayed after 8 days incubation.

Time-course of in vitro antitetanus toxoid antibody production after booster immunization. The in vitro synthesis of antitetanus toxoid antibody by fractionated lymphocytes from nonboosted individuals was undetectable either in the presence or absence of PWM.

The capacity for in vitro antitetanus toxoid antibody

FIGURE 3 In vitro synthesis of antitetanus toxoid antibody and IgG by PBL from four individuals 14 days post-booster immunization. Cultures of 2×10^6 Ficoll-Hypaque-purified lymphocytes were initiated with PWM on day 0. Duplicate cultures were removed at subsequent intervals, and the culture supernate analyzed for antitetanus toxoid antibody (A) and total IgG (B). (---) No PWM.

synthesis developed within 2 wk after booster immunization, reached maximum levels between 3–6 wk post-immunization, and declined by 9 wk (Fig. 4 A). The synthesis of total IgG was constant for the duration of the experiment (Fig. 4 B).

Absence, before boosting, of circulating helper T and B lymphocytes capable of reacting with PWM to synthesize antitetanus toxoid antibody in vitro. Helper T-lymphocyte function, specific for the synthesis of antitetanus toxoid antibody, was evaluated in PBL from individuals before booster immunization by T- and B-lymphocyte co-culture experiments. The T lymphocytes were irradiated (3,000 rad) before use to reduce suppressor T-lymphocyte influences.

After 8 days incubation in vitro with PWM, T- and B-lymphocyte combinations from three individuals boosted 14 days before the experiment synthesized an average of 190 U of antitetanus toxoid antibody (Fig. 5). The combination of B and T lymphocytes from nonboosted individuals consistently synthesized only background levels of antitetanus toxoid antibody. When T lymphocytes (1.6×10^6) from nonboosted individuals were co-cultured with B lymphocytes (0.4×10^6) from boosted individuals, an average of 52 U specific antibody were synthesized. The synthesis of total IgG was unimpaired.

The nonboosted individuals were then immunized and the helper function of the T lymphocyte was reassessed 14 days later with the same B-lymphocyte combinations. The average number of units of antitetanus toxoid antibody synthesis in vitro increased to 177 U.

The functional B-lymphocyte activity for the in vitro synthesis of antitetanus toxoid antibody was similarly evaluated in PBL from nonboosted individuals.

In the presence of PWM B lymphocytes from nonboosted individuals synthesized an average of 20 U of

FIGURE 4 Effects of time after immunization on in vitro antibody and IgG synthesis. Autologous PBL (0.4×10^6 B cells, 1.6×10^6 T cells) from individuals immunized on day 0 were cultured in vitro with PWM at various intervals after immunization. After 8 days in culture, the supernatant fluids were analyzed for the quantity of antitetanus antibody produced (A) or total IgG (B).

antitetanus toxoid antibody when irradiated T lymphocytes from boosted individuals were added. Again, the synthesis of total IgG was normal indicating that T- and B-lymphocyte collaboration for the synthesis of total IgG was unimpaired.

The nonboosted individuals were then immunized, and the ability of B lymphocytes from these individuals to synthesize antitetanus antibody was reassessed 14 days later with the same irradiated T-lymphocyte combination. After booster immunization in vitro level of antitetanus antibody synthesized increased to an average of 160 U. These results indicate that booster immunization produced an increase in the frequency and/or activity of helper T lymphocytes and B lymphocytes capable of being stimulated in vitro with PWM to synthesize antitetanus toxoid antibody.

Functions of B lymphocytes and suppressor T lymphocytes during the decline of in vitro synthesis of antitetanus toxoid antibody. The role of suppressor T lymphocytes in the declining synthesis of antitetanus toxoid antibody in vitro 30–60 days postimmunization was determined by a comparison of the ability of T lymphocytes and irradiated T lymphocytes to collaborate with autologous B lymphocytes in the PWM-stimulated synthesis of the antibody.

The suppression index (S.I.) for both total IgG synthesis and antitetanus toxoid antibody was defined as: $S.I. = (\text{synthesis in presence of T lymphocytes}) / (\text{synthesis in presence of irradiated T lymphocytes})$ and was measured from 14 to 60 days postimmunization. The index of suppression for total IgG varied little for the duration of the experiments and averaged 0.55 (Fig. 6).

FIGURE 5 Function of tetanus toxoid-specific B lymphocytes and helper T lymphocytes before booster immunization. Combinations irradiated (IRR) T lymphocytes and B lymphocytes from individuals preboost (day 0) or different times after boosting (days 14 and 28) were cultured for 8 days, and the amount of antitetanus toxoid antibody and total IgG synthesized determined ($\pm$ SE). d, day.

FIGURE 6 Tetanus toxoid-specific B-lymphocyte and suppressor T-lymphocyte activity after booster immunization. Autologous B lymphocytes (0.4×10^6) and T lymphocytes or irradiated T lymphocytes (1.6×10^6) were co-cultured in vitro for 8 days at different intervals after immunization and the total IgG and antitetanus toxoid antibody determined. The suppression index (S.I.) ($\pm$ SE) was defined as S.I. = (synthesis in presence of T lymphocytes)/(synthesis in presence of irradiated T lymphocytes). Brackets indicate intervals over which samples were pooled. (○) Total IgG; (●) antitetanus toxoid antibody. The maximum units of antitetanus toxoid antibody ($\pm$ SE) are given in brackets beneath each point.

At 14 days postimmunization the index of suppression for the synthesis of antitetanus toxoid antibody was 0.60. This declined to <0.1 by day 50, suggesting an association of increased specific T-cell suppression with the declining synthesis of antitetanus toxoid antibody.

The maximum synthetic activity of antitetanus toxoid-specific B lymphocytes was also evaluated at different intervals postimmunization. This parameter was expressed as units of antibody synthesized by 0.4×10^6 B lymphocytes in the presence of 1.6×10^6 autologous irradiated T lymphocytes and is shown in brackets below the suppression index in Fig. 6. The maximum B-lymphocyte activity peaked at approximately day 30 and slowly declined, approaching baseline levels after 60 days. Similar results were obtained when allogenic T lymphocytes obtained 14–35 days postimmunization were substituted for autologous T lymphocytes, indicating that the decreased B-lymphocyte activity was not due to insufficient numbers of helper T lymphocytes. Thus both increased T-lymphocyte suppression and decreased B-lymphocyte function appear associated with the decline in the in vitro synthesis of antitetanus toxoid antibody at times >30 days postbooster immunization.

DISCUSSION

Previous experiments using the PWM-driven in vitro synthesis of Ig by human PBL have demonstrated the existence of functionally and antigenically defined sub-

populations of regulatory T lymphocytes which modulate the synthesis of Ig by B lymphocytes. However, the roles of these circulating regulatory lymphocytes in the in vivo homeostasis of antibody synthesis have not been clearly defined. Three major conclusions can be drawn from the results presented here which directly relate to this question: (a) The ability of PBL to respond in vitro to PWM by the production of antitetanus toxoid antibody is dependent upon recent booster immunization. (b) Before booster immunization, there are insufficient quantities of both B lymphocytes and helper T lymphocytes capable of being stimulated by PWM to produce detectable antitetanus toxoid antibody in vitro. (c) An increased number, or activity, of circulating radiation-sensitive inhibitory T lymphocytes as well as decreased activity of B lymphocytes are associated with the decline in the in vitro synthesis of antitetanus toxoid antibody at protracted times after booster immunization.

At first these findings appear to be in contrast to other investigators' results. Before booster immunization with tetanus toxoid, Pagleroni and Mackenzie (24) demonstrated that lymphocytes capable of binding radiolabeled tetanus toxoid circulated in the blood with a frequency of 8/5,000 total lymphocytes. This is within the range of antigen-binding cells detected in the spleens of mice and guinea pigs after immunization with a variety of antigens (25, 26). These cells most likely represent B lymphocytes because of their multiplicity of high affinity receptors (27). Additional evidence for the existence of tetanus toxoid B lymphocytes in the circulation of primed (not boosted) individuals has come from the work of Geha et al. (28) who demonstrated that in the presence of a tetanus toxoid-specific T-cell factor, B lymphocytes from nonboosted individuals produce antitetanus toxoid antibody in vitro. Our results, however, have shown that an antitetanus toxoid antibody response cannot be initiated by PWM in vitro before an in vivo booster immunization. This would imply either a lack of, or insufficient numbers of, tetanus toxoid recognative B lymphocytes that are reactive with PWM. The radioimmunoassay we employ is capable of detecting the specific antitetanus toxoid antibody synthesized by the plasma cell progeny of one to three (depending on division time) B lymphocytes over an 8-day incubation. Our cultures contain 4×10^5 B lymphocytes, and we should therefore be able to detect the synthesis of antitetanus toxoid antibody in cultures of PBL where the frequency of tetanus toxoid-specific B cells would be $\approx 1/1 \times 10^5$. As tetanus toxoid-binding lymphocytes circulate with a frequency of $\approx 1/1,000$ B cells, it would suggest that the majority of these cells do not respond to PWM in vitro before recent antigen exposure by booster immunization.

PWM stimulation of immunoglobulin synthesis is modulated by T lymphocytes, and it is possible that booster immunization is required to generate sufficient numbers of T lymphocytes which may promote the synthesis of antitetanus toxoid antibodies *in vitro*. However, the existence of circulating antigen-specific T lymphocytes before booster immunization can be inferred from the studies of McMichael et al. (29) and Sasazuki et al. (30) who demonstrated a significant *in vitro* proliferative response of human PBL from nonboosted individuals when cultured with tetanus toxoid. This proliferative response has been shown by Chess et al. (31) to be mainly a T-cell function. Additionally, Geha et al. (28) have generated a factor from T lymphocytes that can induce the synthesis of IgG antitetanus toxoid antibody when added to primed B lymphocytes *in vitro*. This factor is generated *in vitro* by stimulating T lymphocytes with tetanus toxoid. Thus, although tetanus toxoid-binding B lymphocytes, and at least some tetanus toxoid-reactive T lymphocytes, may be present in the circulation of nonboosted individuals, PWM does not elicit an *in vitro* antitetanus toxoid antibody response before booster immunization.

In contrast to our findings, the production of anti-sheep erythrocyte antibodies (IgM) *in vitro* after PWM stimulation occurs without apparent *in vivo* booster immunization (32, 33). It would appear, however, that anti-sheep erythrocyte antibodies are present in the serum of individuals who have not been immunized (34) and may be the result of a recent immune response to cross-reacting antigens. If this were the case then known booster immunization might not be required to generate an anti-sheep erythrocyte response with PWM. It would appear, in fact, that a large number of clones of different antibody-producing lymphocytes, responsive to PWM, exist in the circulation at any given time. Even at the peak of the antitetanus toxoid antibody response after booster immunization, specific antitetanus toxoid antibody accounts for only $\approx 3\%$ of the total IgG synthesized *in vitro*. Thus, the potential for the synthesis of cross-reacting antibodies to a complex antigen such as sheep erythrocyte would seem reasonable.

Studies on the *in vitro* generation of antibody by antigen stimulation have also shown no requirement for recent booster immunization (16, 32, 35) although there may be requirement for at least one prior immunization (28). These results can be reconciled with ours by viewing the lymphocytes in the circulation as existing in functional compartments depending on the interval since booster immunization. Before booster immunization, tetanus toxoid-specific B and T lymphocytes circulate which will respond to antigen but which are unresponsive to PWM. The perturbation of the immune system by booster immunization results in a

transient state of increased tetanus toxoid-specific lymphocyte number and(or) activity in the circulation capable of responding to PWM as well as to antigen. This activity remains in the circulation until approximately 60 days postimmunization. A comparison of the quantities of total IgG and antitetanus toxoid antibody produced by B lymphocytes in the presence of T lymphocytes or irradiated T lymphocytes demonstrated progressive and selective antitetanus toxoid antibody suppression occurring from 14–60 days postimmunization. These results suggest that PWM-stimulated suppression of Ig synthesis may occur by polyclonal activation of antigen-specific suppressor T lymphocytes whose *in vivo* role is to limit the extent of the immune response. A similar demonstration of antigen-specific T lymphocytes has been reported by Uytend Haag et al. (36). Decreased B-lymphocyte reactivity also appears associated with the decline in the amount of antitetanus toxoid antibody synthesized *in vitro* at long intervals after immunization.

Finally, our results may give an indication of the action of PWM on circulating lymphocytes. The stimulation of Ig synthesis by PWM involves the collaboration of B and T lymphocytes. This collaboration may be viewed either as a nonspecific stimulation of T lymphocytes which subsequently activate all clones of B lymphocytes, or alternatively, as a polyspecific stimulation involving the interaction of specific clones of T and B lymphocytes. Before booster immunization, the level of T-lymphocyte help specific for the synthesis of antitetanus toxoid antibody was low and increased fourfold after booster immunization. This result suggests that booster immunization increases the number and(or) activity of antigen-specific circulating helper T lymphocytes which are capable of collaborating with tetanus toxoid-recognitive B lymphocytes in the synthesis of antitetanus toxoid antibody in the presence of PWM but in the apparent absence of antigen. We would postulate the generation of PWM-reactive lymphocytes by booster immunization results from the *in vivo* priming with antigen of the B and(or) helper T lymphocytes so that they become capable of specific recognition and collaboration in the absence of additional antigen. Thus, PWM stimulation of antibody synthesis *in vitro* may be truly polyclonal (i.e., multispecific) as opposed to nonspecific. Experiments are in progress to further explore this question.

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