Deregulation of Mouse Antibody-Forming Cells In Vivo and in Cell Culture by Streptococcal Pyrogenic Exotoxin

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An unregulated, elevated rebound of antibody levels in rabbits was shown to follow late (10 to 15 days) after streptococcal pyrogenic exotoxin (SPE)-induced immunosuppression. Because of that result we have suggested that SPE acts by preferentially inhibiting a regulatory cell which normally limits the extent of full expression of antibody formation by B-cells. We are currently testing this hypothesis in mice. NIH (Swiss Webster) mice (+/+ or NIH (Swiss Webster) mice heterozygous (+/nu) for the mutant athymic nude gene and phenotypically normal showed an elevated plaque-forming cell (PFC) response to sheep erythrocytes (SE) late (10 to 15 days) after immunosuppressive SPE treatment similar to that described in rabbits. Homozygous nude mice (nu/nu) that are phenotypically athymic normally show a reduced early (4 day) PFC response to SE (a T-cell-dependent antigen) as compared with +/nu littersmates or +/+ parent strain mice. This cryptic early 4-day response was improved by injection of purified endotoxin (a B-cell mitogen), but these relatively elevated nude PFC responses had decreased to normal control (SE only) nude PFC levels before 10 days. In similar SE-injected nude mice treated instead with SPE, no elevation at 4 days was observed and, more pertinently, the late (10 to 15 day) elevated rebound of PFC levels observed in normal response controls (+/nu or +/+ ) was not observed. Similar experiments were subsequently conducted in Marbrooktype spleen PFC cultures during periods of 12 days. The results of these experiments paralleled the in vivo results above, and in addition showed that SPE induced a large proliferation of either +/+ or +/nu cells (T- and B-cells) in culture but had no such effect on nu/nu cells (B-cells) in culture. Purified endotoxin, the B-cell mitogen, had a better sparing effect on nu/nu cells in this respect. These results are consistent with our premise that SPE inhibits preferentially the function of a regulator of the antibody response. The regulator appears to be a T-cell and is likely a suppressor T-cell.

The finding of an elevated antibody level to a single antigen exposure in rabbits late after streptococcal pyrogenic exotoxin (SPE)-induced immunosuppression has been documented recently (12). This result led us to speculate that SPE may preferentially inactivate a regulatory cell. Since elevated plaque-forming cell (PFC) levels were measured, this effect is not thought to be mediated at the catabolic level of immunoglobulin (Ig) degradation. We showed earlier that SPE effects phagocytosis in the intact rabbit (10) and that it is suppressive for early 4-day response PFC (11). Current data obtained with the mouse and with congenitally athymic mice (25) that respond poorly to T-dependent antigens (1, 19) have concentrated our attention on a regulatory cell, possibly a suppressor T-cell (3, 8, 9, 24), as the preferential site of action of the toxin, as previously speculated (12).

MATERIALS AND METHODS

Mice. National Institutes of Health (NIH) strain, Swiss-Webster [NIH(SW)], NIH(SW)nu/nu ("nude"), and NIH(SW) +/nu mice, 6 to 12 weeks old and of either sex, were obtained from the NIH Small Animal Section. The nu/nu mice were kept in disposable cages on sterilized bedding. Food holders and water bottles were presterilized. The mice were given acidified, chlorinated water; otherwise they were handled routinely while in our animal rooms. Injections were made intravenously (i.v.) by tail vein using 27-gauge needles. Littersmates of nu/nu mice with coats are +/nu and the parent strain NIH (SW) mice are designated +/+.

Sheep erythrocytes. Sheep erythrocytes (SE) were prepared from sterile sheep blood which was received...
weekly in donor bottles containing acid citrate solution from the Ungulate Unit, NIH Animal Center. For SE immunogen, gravity-packed SE were resuspended in cold, sterile 0.1 M tris(hydroxymethyl)aminomethanes-buffered saline (TBS), resedimented by centrifugation two times at 1,000 x g and finally suspended in a volume of TBS to give the desired number of SE per milliliter. The injection volume for mice was 0.2 ml. For culture immunogen, Hanks balanced salt solution (HBSS) was substituted for TBS as the washing and suspension medium. All other SE suspensions were prepared by the first procedure, except that the final suspension of reagent SE was in modified TBS (TBS + 0.0007 M CaCl2, 2H2O, 0.004 M MgCl2, 6H2O, and 0.056 M dextrose).

Spleen cell suspension. There were two types of spleen cell suspension (SC). Spleens were obtained by aseptic surgery and immersed immediately into cold HBSS held on ice. When obtained from immunized mice for immediate assay of PFC, the spleens were quartered and pressed against 0.45-μm stainless-steel, wire-mesh cloth (W. S. Tyler Co., Mentor OH 44060). The single-cell suspensions were obtained by washing the cells through with HBSS and collecting them in a beaker. The SC were then sedimented by centrifugation at 1,000 x g and resuspended in HBSS to an appropriate cell number for plating. To obtain culture-seeding SC suspensions, spleens obtained as above were sectioned, and the cells were gently teased from the membrane in HBSS held on ice in an appropriate size sterile plastic petri dish using iris scissors and forceps. The cells were dispersed further by jetting through the orifice of Pasteur pipettes. The cells were sedimented once by centrifugation at 1,000 x g and resuspended in a volume of nonsupplemented culture medium for counting.

Cell counting. SE and SC counts were performed electronically in the Autocytometer-II (Fisher Scientific, Pittsburgh, Pa.).

Cell cultures. SC prepared as described above were cultured in Marbrook vessels (21) (Bio-Research Glass, Inc., Vineland, N.J.) on a dialyzing membrane suspended in excess culture medium. The culture medium was alpha minimal essential medium (27), modified Eagle minimal essential medium (Flow Laboratories, Inc., Rockville, Md.) supplemented with 10% fetal bovine serum obtained from either Gray Industries, Inc., Ft. Lauderdale, Fla., Microbiological Associates, Bethesda, Md., or Rhes Chemical Co., Kankakee, Ill. Cell cultures were seeded usually at 20 x 10⁴ acid-resistant cells (leukocytes) per ml of culture. Inoculated cultures received 1.5 x 10⁴ to 2.5 x 10⁴ SE as a 0.04-ml drop in HBSS at the start of cultures. Other additions, e.g., toxins, were added at the concentrations given in the text in volumes of 0.1 or 0.2 ml immediately after cells were seeded. Penicillin (100 U/ml) and streptomycin (100 μg/ml) were added to all cultures. Cultures were incubated in a humidified tissue culture incubator at 37 C in a continuously flowing atmosphere of 83% N₂, 7% O₂, and 10% CO₂. The cultures were rocked continually on a rocker platform at 5 to 7 cycles per min, since contrary to our expectations (21), preliminary tests showed that the rocking procedure was beneficial when compared with static cultures. Cells were harvested from the membrane and inner cylinder by use of Pasteur pipettes, sedimented by centrifugation at 1,000 x g, resuspended in 1.0 ml of HBSS, assayed for PFC, and counted; thick smears were prepared and fixed for staining. The smears were stained with Giemsa or Wright stain.

PFC assays. Molten agarose (pretested lot no. 81C-2680, Sigma Chemical Co., St. Louis), 2.5 ml of a 0.8% solution in 0.1 M tris(hydroxymethyl)aminomethane-buffered Eagle medium held at 47 C, was mixed with 0.2 ml of a washed 20 to 50% suspension of SE in modified TBS and portions (e.g., 0.1 and 0.2 ml) of appropriate dilutions of the SC suspension. This mixture was poured immediately into plastic tissue culture dishes (20 mm by 100 mm), held at room temperature on a leveling board, and allowed to gel. The plates were then incubated for 1.5 h at 37 C. To estimate IgG-secreting PFC by the indirect assay (7, 28), designated plates were flooded with 4.0 ml of an appropriate dilution in modified TBS of hyperimmune, SE-absorbed, rabbit anti-mouse hyperimmune, anti-SE antibodies and incubated for an additional 1 h. Those plates designated for estimation of 19S IgG-secreting PFC by the direct assay (13, 14) were flooded with modified TBS and incubated concurrently. For plaque development all plates were rinsed briefly with modified TBS, flooded with 4.0 ml of 10% guinea pig complement (BBL, BioQuest, Cockeysville, Md.), and incubated at 37 C for an additional 1 h. Plates were cooled at 4 C and stored briefly or scored immediately for plaques. The plaques were counted at 7 x to 15 magnification, using a dark-field stereomicroscope (Olympus Optical Co., Tokyo). IgG plaque counts represent the total PFC per spleen as estimated from antoglobulin-facilitated indirect plates.

SPE. This was the group A streptococcal exotoxin isolated and purified by Kim and Watson (18) and used previously (11, 12). It was produced by type 10, strain NY-5, group A streptococcus. It is an acidic protein with an average molecular weight of 29,000. Its relative biological activity, using the minimal pyrogenic dose (MPD-3), was 0.07 μg/kg i.v. in rabbits (11, 12, 18). SPE was dissolved in TBS for injection in fetal bovine serum-free culture medium when added to SC cultures.

Et. Endotoxin (Et) was the purified hot phenol-water-extracted (17, 30) lipopolysaccharide from Salmonella typhimurium L5, wild type, a gift from Y. B. Kim. Relative biological activity using the MPD-3 was 0.0024 μg/kg i.v. in rabbits (17).

Et was dispersed in water at 100 C as a concentrated solution and then diluted appropriately in TBS for injection or in fetal bovine serum-free culture medium for addition to SC cultures. The mean lethal doses of Et and SPE in mice were 350 and 3,600 μg, respectively (17, 18).

RESULTS

Effect of SPE on the mouse anti-SE PFC response. We observe that SPE affects mouse PFC as well as those of rabbits (Fig. 1). SPE injected into mice 3, 24, and 48 h after a single
Effect of SPE on the mouse anti-SE PFC response in culture. Figure 2 shows results obtained in Marbrook-type (21) PFC cultures, which were inoculated with SE and treated with SPE or with the B-cell mitogen Et (2) at the time of culture initiation. Et elevated the early 4-day peak of PFC slightly above SE-inoculated controls, consistent with its B-cell stimulating activity (2). The early 4-day response in SE-inoculated, SPE-treated cultures was suppressed (≈ threefold) but was greatly elevated (≈ eightfold) late at 12 days, a time when SE-inoculated controls and Et-treated, SE-inoculated, control culture PFC levels had decreased. Thus, the effect characterized by an early suppression followed by recovery and a late increase above controls can be observed.

Injection of SE caused a slight (≈ twofold) suppression at 4 days of the number of anti-SE PFC developed as compared with the number of such PFC developed in control mice injected with SE alone (10.017 ± 923 PFC/spleen versus 18,810 ± 877 PFC/spleen, respectively). This level of PFC suppression in mice was less than that observed in similar previous experiments conducted in rabbits (11). Nevertheless, the 19S Ig PFC response measured at 12 days in SPE-treated mice was maintained 10-fold higher than that of control mice injected with SE alone. Moreover, mice treated similarly with the B-cell mitogen, Et, showed an early elevation of 19S Ig PFC, a response consistent with expectations for one modified by a B-cell mitogen and the reverse of the response observed after SPE treatment.

IgG PFC in SPE-treated mice were markedly elevated in comparison with those in either Et-treated mice or the nontreated control mice at 12 days. Thus, the "unregulated" increase of PFC which occurs late (11) can be studied in the mouse as well as in the rabbit. Moreover, the T-cell, B-cell synergy was originally observed in mice (6) and more elementary information and methodology is available in this species. Of particular interest here was that nude, congenitally athymic mice were available.

Fig. 2. Effect of SPE (100 μg/culture) on the anti-SE (1.5 × 10⁶ SE/culture) PFC response in SC (2.75 × 10⁶ seeded) cultures from NIH (SW) mice. Symbols: (□—□—), (uninoculated) SE alone for 12 days assayed for anti SE, PFC at 4, 8, or 12 days; (○—○) SE inoculated, assayed for anti-SE, PFC at 4, 8, or 12 days; (△—△), SE inoculated, Et treated (10 μg/culture), assayed for anti-SE, PFC at 4, 8, or 12 days; (□—○), SE inoculated, SPE treated, assayed for anti-SE, PFC at 4, 8, or 12 days. Each point represents the PFC in a pool of 3 from duplicate or triplicate cultures in a representative experiment. Distinctive IgG PFC could not be ascertained from culture.
even more clearly in cell culture. Moreover, a very similar effect (Fig. 3) was exhibited by SPE- and Et-treated unincoculated cultures. This delayed SPE effect on background PFC makes it apparent that the background anti-SE-PFC clones can also be released from a normal regulatory influence. The early (4 day) background PFC elevation in Et-treated cultures is consistent with previous observations in the rabbit (11), an effect originally described by Johnson et al. (15, 22). Since it is now held that Et is a preferential B-cell mitogen (2), the effect of Et here is probably due to a direct stimulatory effect on existing B-cell background clones.

**Increased cell number in SPE-treated cultures.** SPE-treated cultures showed a 120% increase in cell number at 4 days (Fig. 4); the number was essentially maintained for 8 days and was 85 to 90% maintained at 12 days in both SE-inoculated and unincoculated SPE-treated cultures. This effect was not observed in Et-treated cultures, although Et-treated cultures were better maintained (60 to 70%) than SE-inoculated control cultures and cells alone in culture. The appearance and relative number of these cells at 12 days are shown in Fig. 5. Unincoculated cultures (lower left panel) and SE-inoculated, nontreated cultures (lower right panel) showed degenerating nondividing cells only, and is consistent with the data of Fig. 4. Cultures treated with SPE or with Et showed a much larger number of basophilic intact lymphocytes with only a few degenerating cells. Similar morphological criteria were presented recently as evidence of cell proliferation (26). Consistent with the data of Fig. 4 was the observation (Fig. 5) that SPE-treated cultures showed the greatest number of intact lymphocytes with good basophilia at 12 days and they appeared to be a more homogeneous population.

**Effect of SPE on the anti-SE PFC response of athymic nude mice.** The anti-SE PFC response of nude mice (congenitally lacking T-cells) (Fig. 6, top panel) was 10- to 15-fold (1.0 to 1.5 logs) lower than the +/nu, T- and B-cell control response (bottom panel) in those groups injected with SE alone at all assay points. Whereas Et stimulated an increased PFC level in nude mice (top panel, 4 and 8 days), it was surprising that SPE did not induce suppression of the early (4 day) nude PFC response. This observation is being investigated further. More relevant to our current interpretation, however, was that the characteristic delayed "unregulated" increase of PFC at 10 to 15 days did not occur in nude mice (Fig. 6, top panel). Nude mice did not exhibit IgG PFC regardless of the stimulus in these experiments, whereas the unregulated response, post-SPE treatment, was accentuated in the IgG PFC compartment of +/+ mice (Fig. 1). The responses of +/nu littermate mice (Fig. 6, bottom panel), in a concurrent control experiment, were characteristic of SPE-treated parent strain +/+ mouse responses. These characteristics as shown by +/nu mice (Fig. 6, bottom panel) were: SPE suppression or delay at 4 days and elevation of IgG PFC which occurred late (12 to 14 days) in the SPE-treated group.

**Effect of SPE on the athymic nude mouse anti-SE PFC response in culture.** Et, a B-cell mitogen, stimulated the 4-day peak response of
nude (B-cells) PFC (Fig. 7). This was evident in both SE-inoculated (SE + Et) and background (uninoculated) (Et only) cultures. Consistent with previous data, SPE-treated (SE inoculated and uninoculated) nude culture responses were lower than the control inoculated-culture response and much lower than those stimulated in addition with the B-cell stimulant Et. The more important finding in this experiment was that the unregulated delayed PFC increase in SPE-treated cultures did not occur, thus contrasting sharply with the counterpart experiment in +/- mouse SC cultures (Fig. 2). Moreover, SPE-treated cultures did not show cell proliferation at 4 or 12 days, but rather a steady decline in cell number (Fig. 8). It is pertinent, however, that Et-treated nude (B-cell) cultures were maintained at a higher cell number at both points tested.

DISCUSSION

We have previously documented an unexpected finding of elevated antibody formation after suppressed antibody formation induced with streptococcal pyrogenic exotoxin (12). This paper presents recent data obtained in a mouse model system in support of the speculation that SPE might have a preferential effect on a regulator cell. Temporary elimination of the regulator cell or inhibition of its activity would
allow clonal expansion of B-cells, among which would be greater elevated levels of antigen-stimulated descendent PFC (Fig. 1, 2, and 6). It might be expected that background PFC populations (unstimulated B-cells) may also be unleashed by SPE. Results consistent with this were obtained in spleen cell cultures of +/- mice containing both B- and T-cells (Fig. 3). The counterpart experiment involving nu/nu cells (Fig. 7) moreover, supports this conclusion since Et-treated cultures showed that these cells were capable of being stimulated (i.e., direct B-cell stimulation), but SPE with suggested preferential antisuppressor T-cell activity was without effect (i.e., no regulator available). In addition, this experiment showed that SPE had only little overt effect on B-cells alone, at the dose used in these experiments, and whether the early (4 to 8 day) suppressive effect of SPE is mediated through an effect on helper T-cells (6) has not yet been determined. Baker et al. (4) have recently offered strong evidence in a helper T-cell-independent antigen (SSS-III) system that suppressor T-cells modulate B-cell maturation and clonal expansion. We have not yet tested the effect of SPE on the response to T-independent antigens nor on a synthetic hapten-carrier immunogen, two systems which should provide interesting future data relative to any effect of SPE on helper T-cells (6, 23, 29). However, available data predict that the response to these antigens would have similar
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characteristics to that shown currently. SPE would act to retard the regulator step, leaving B-cells unregulated or available for amplification by amplifier cells as suggested by Baker et al. (3), in the case of T-independent antigens, or by T-helper cells (6), in the case of synthetic hapten-carrier, T-dependent immunogens (23). The mode of action of SPE being tested (Fig. 9) would require only a slight modification should it be shown that suppressor activity is another activity of the T-helper cell or of this cell at a different developmental stage (5, 16).

The finding of a neutral or negative effect of SPE in nude mice, which are T-cell negative but not known to be macrophage deficient, argues against a remotely possible suppressor-macrophage as the SPE target. This, however, is important to be ruled out in the future since SPE in a different form was shown in our very early studies of its biological activity to be antiphagocytic (10), although the type of phagocyte effected, polymorphonuclear or mononuclear, is not known. It is, nevertheless, less remotely possible that an early coordinate suppressive effect could be mediated by macrophages possibly by withholding effective antigen and that this suppression could be slowly relieved by an antiphagocytic effect of SPE.

Because of a current limited amount of purified SPE, we have not yet determined how long the unregulated PFC levels are retained. In the rabbit experiments, however, the persistence of the unregulated PFC level was dose dependent, and a high-dose-treated group (70 μg/day, 3 days) showed elevated levels which were maintained longer than 30 days (12). This is consistent with our suggestion (12) that the suppressor cell population recovers more slowly than does suppressed B-cells and possibly therefore may be sufficiently affected so as to interfere with induced and constituent states of tolerance. In this respect, we do not yet know whether a similar sustained "unregulated" antibody response to a second or a battery of antigens would be simultaneously deregulated or not. This important possibility is being tested. Relative to the attributes of SPE to the disease-producing process of group A pathogenic streptococci, we have previously suggested that the early transient suppression may be relevant to acute streptococcal disease by creating a parasite-preferring, low-antibody environment for the streptococcus (3). Secondly, a number of the late streptococcus-associated sequelae have not been consigned to immediate direct effects of the streptococcus but are considered in the realm of autoimmunity (20) somehow associated with or initiated by the streptococcus. In this respect, whether SPE can effect self-tolerance adversely may be tested in specific animal model systems in the future.

Presented in Fig. 9 is a diagram illustrating how SPE might act. B-cells, when stimulated by antigen and amplified by helper T-cells, initiate maturation and proliferation of a clone of B-cells. Clone size normally would be regulated by a suppressor cell. SPE would affect inhibition of the suppressor event, thus allowing full unregulated expression of B-cell clonal potential possibly with additional amplification by helper cells. The diagram allows for the possibility that the suppressor cell and the helper cell may be different stages of the same cell line (suggestions contained in references 5 and 16). This speculated mechanism by which SPE may deregulate antibody formation, by

Fig. 9. Illustration of how SPE might deregulate antibody formation by interference with a normal regulator of B-cell clonal expansion. The suppressor cell (s-T) would normally limit the extent of B-cell proliferation. SPE would either preferentially inactivate or inhibit the suppressor cell. The B-cell would then be free, stimulated by specific antigen, and amplified by the helper T-cell into an enlarged clone of antibody-secreting, mature antibody-forming cells (B-c).
affecting deregulation of progenitor antibody secreting B-cell clones, if further supported, will define important heretofore unrecognized attributes of SPE. Furthermore, SPE may be a valuable probe for further studies on regulation of the antibody response.

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LITERATURE CITED