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Immunity and Responses of Circulating Leukocytes and Lymphocytes in Monkeys to Aerosolized Staphylococcal Enterotoxin B

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Rhesus monkeys immunized intramuscularly or orally with staphylococcal enterotoxin B (SEB) toxoid or SEB toxoid incorporated in microspheres made of poly(DL-lactide-co-glycolide) were challenged with a lethal dose of aerosolized SEB to study their immunity and cellular responses in the circulation. It was found that circulating antibodies play a critical role in preventing SEB from triggering toxicosis. Monkeys with high levels of antibodies survived, while those with low levels underwent 2 to 3 days of toxicosis and died. Intramuscular immunization induced high levels and oral immunization induced low levels of antibodies. The circulating antibodies in surviving monkeys decreased dramatically within 20 min and started to rebound at 90 min after SEB challenge. At 90 min, the dying monkeys showed in the circulation a dramatic increase of polymorphonuclear leukocytes and decreases of NK cells and monocytes (CD16 and CD56 markers) as well as of lymphocytes with HLA-DR, CD2, CD8, and IL2Rα (CD25) markers. The number of polymorphonuclear leukocytes showed an inverse correlation with the numbers of monocytes and various lymphocyte subpopulations which, except for IL-2R, CD16, and CD56(+) cells, showed a direct correlation with one another. The changes in the populations of leukocytes, monocytes, NK cells, and lymphocytes seem to be an indication of initial toxicosis; however, the roles of these cells in toxicosis and death remain to be defined.

Staphylococcal enterotoxin B (SEB) causes food poisoning when ingested and toxic shock when it enters the blood circulation and affects systemic tissues (3, 6, 10, 16, 19, 39, 44). The pathological mechanisms of toxicosis and death are still largely unknown. SEB, a superantigen, binds to class II histocompatibility antigens on antigen-presenting cells, and the SEB-class II antigen complex is then presented to and activates T cells bearing certain T-cell antigen receptor Vβ elements (11, 22). It has been suggested that activation of both the antigen-presenting cells and T cells results in the production of large amounts of monokines and lymphokines, which cause the disease (21, 26). On the other hand, other cell types may also be involved. Scheuber and coworkers (37, 38) have shown that SEB injected intradermally into cynomolgus monkeys results in an immediate-type skin hypersensitivity reaction which is caused mostly by degranulation of mast cells. This mast cell degranulation may be caused by a direct activation by SEB of mast cells or of nerve cells which release neuropeptides, which then activate mast cells (1). Histamine H2 receptor antagonists can block SEB-induced emesis and skin reactions (37). Recently, we have also shown that SEB can induce serotonin release from cultured mast cells (23).

If SEB intoxication is caused by activated macrophages, T cells, and other cell types and their mediators, the toxicosis could be a cascade and/or a combination of sequential pathophysiological changes among functionally interrelated tissues. A blockade of the initial phase in which SEB reacts with the target cells should result in effective immunity. However, immunity to SEB and mechanisms for protection in the early phase of SEB intoxication are still not clearly defined. Classical studies on immunity to SEB focused on the ability of circulating antibodies to prevent the symptoms of food poisoning and death from toxic shock in rabbits and monkeys (5, 6, 28, 40). Large amounts of circulating antibodies were able to prevent intravenous SEB-induced toxic shock and death but were unable to consistently prevent oral SEB-induced food poisoning.

SEB is the major staphylococcal enterotoxin closely associated with nonmenstrual toxic shock syndrome and accounts for the vast majority of nonmenstrual toxic shock syndrome cases that are not caused by toxic shock syndrome toxin 1 (39, 44). Most of these toxic shock cases are generally due to an infection with enterotoxin-producing staphylococci, which reach the deep tissues via the opening of wounds and injuries (16, 39, 44). In recent years, however, cases of nonmenstrual toxic shock syndrome have been found in staphylococcal infections of mucosal tissues, particularly in cases of barrier contraceptive usage and secondary infections in the respiratory tract of influenza patients (16, 25). Because antibiotic-resistant and toxin-producing staphylococci are widely present in the body, particularly in the nose and throat, respiratory toxicosis and toxic shock may become a serious, if not unmanageable, problem during outbreaks and epidemics of respiratory infectious diseases.

With the increasing problem of respiratory toxic shock on the one hand and lack of knowledge concerning protective immunity on the other, we have sought strong SEB vaccine candidates and studied their ability to protect against toxicosis caused by SEB aerosol as well as the mechanism of

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protection. It is well known that SEB toxoid is a relatively good immunogen in inducing high-titered antibodies in monkeys and rabbits (5, 40, 43), and, recently, SEB toxoid-containing microspheres made of poly(DL-lactide-co-glycolide), a nontoxic biodegradable adjuvant, have been shown to be capable of inducing long-lasting, high-titered antibodies in mice (14). We have used these forms of toxoid to immunize rhesus monkeys and have studied the protective immunity and early responses of the monkeys to aerosolized SEB. We show here the importance of circulating anti-SEB antibodies in protection against toxicosis and death. The antibodies appear to prevent the initiation of SEB toxicosis that is manifested by the responses of circulating leukocytes, monocytes, and lymphocytes. The initial phase of SEB toxicosis, however, seems to be a complicated process which involves interactions of leukocytes, monocytes, and various lymphocyte subpopulations.

MATERIALS AND METHODS

Reagents. Monoclonal antibody reagents against human leukocyte and lymphocyte surface markers were purchased in a Simultest IMK kit from Becton Dickinson Immunocytometry Systems, San Jose, Calif. The monoclonal antibodies were conjugated with either fluorescein isothiocyanate or phycoerythrin. Although all monoclonal antibody reagents stained blood leukocyte, monocyte, and lymphocyte subpopulations from human volunteers, only antibodies against HLA-DR, CD16, CD56, and CD8 markers satisfactorily stained the corresponding monkey cell populations. Monoclonal anti-IL2Rα (CD25) and anti-T11 (CD2) were purchased, respectively, from Becton Dickinson and Coulter Corp., Hialeah, Fla. Rabbit antisera specific for monkey the immunoglobulin A (IgA), IgG, or IgM isotype were purchased from Nordic Immunological Laboratories, Capistrano Beach, Calif. The specificities of the anti-isotype antisera were further confirmed by enzyme-linked immunosorbent assay (ELISA), using purified monkey IgA, IgG, and IgM. The monkey IgA was isolated from pooled monkey serum by affinity chromatography, using a Jacalin lectin column (34). The monkey IgG was prepared by ammonium sulfate precipitation followed by DE-52 chromatography, and the IgM was prepared by ammonium sulfate precipitation followed by Sepharose 4B gel filtration, DE-52 chromatography, and Jacalin lectin affinity chromatography. The rabbit anti-isotype sera, however, contained a small amount of natural anti-SEB antibodies, which were mostly removed by affinity chromatography by using a Sepharose 4B column conjugated with SEB. Goat anti-rabbit immunoglobulins conjugated with peroxidase was purchased from Zymed Laboratories, Inc., South San Francisco, Calif. SEB prepared by the method of Schantz et al. (36) was obtained from the U.S. Army Medical Research Institute of Infectious Diseases, Ft. Detrick, Frederick, Md. SEB toxoid was prepared by the procedure of Warren and coworkers (43) at pH 7.5 and precipitated with alum by John Eldridge in the Department of Microbiology, University of Alabama, Birmingham. The SEB toxoid was incorporated into poly(DL-lactide-co-glycolide) microspheres, which were prepared by the Southern Research Institute, Birmingham, Ala., under a research contract.

Animal care and use. The experiments performed with monkeys strictly adhered to the 1985 Amendments to the Animal Welfare Act (7 U.S.C. 2131, et seq., Army regulation AR 70-18, and Public Law 99-198) and to the "Guide for the Care and Use of Laboratory Animals" (13a) as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, and adopted by the Laboratory Animal Care and Use Committee of our research institutes.

Immunization. Young male and female rhesus monkeys (*Macaca mulatta*) weighing 2 to 3 kg were immunized by injection into the thigh muscle of three doses of 100 μg of SEB toxoid with alum per monkey or two doses of microspheres containing 100 μg of toxoid at 7-week intervals between doses. Oral immunization was performed also at 7-week intervals in Telazol-anesthetized monkeys by stomach intubation with three doses of SEB toxoid (10 mg per dose) in 0.7% sodium bicarbonate buffer or with two doses of SEB toxoid-containing microspheres (100 μg of SEB toxoid per dose). All of the priming and booster immunizations were performed in John Eldridge's laboratory under a research contract. The monkeys immunized with SEB toxoid were shipped to the U.S. Army Medical Research Institute of Infectious Diseases the day following the last booster immunization, while the monkeys immunized with toxoid-containing microspheres were shipped 7 weeks later. Three to 4 weeks after arriving there, the monkeys were challenged with aerosolized SEB. The immunization regimens and challenge date were chosen with the purpose of testing the efficacy of SEB toxoid and toxoid-containing microspheres as vaccines.

Aerosol challenge. One week before SEB challenge, the monkeys were bled under Telazol anesthesia. Blood samples were collected, and plasma samples and leukocytes were taken for determinations of anti-SEB antibody levels and leukocyte and lymphocyte subpopulations. At the time of challenge, monkeys were weighed and anesthetized with Telazol, placed in a head-only exposure chamber modified from a Henderson aerosol apparatus, and individually exposed for 10 min to a lethal dose of approximately 190 μg of SEB aerosol per kg of body weight. Subsequently, the chamber was flushed with air for 5 min. Prior to exposure, the aerosol machine was calibrated and the amount of SEB for each monkey was estimated on the basis of body weight. The monkey was then exposed to the SEB aerosol, and a sample of SEB was taken and quantified to ascertain the dose. After the machine was flushed with air, monkeys were removed from the chamber, and blood samples were collected 20 and 90 min later. Monkeys were then allowed to rest and recover in their own cages and were observed closely around the clock for clinical symptoms for the next 10 days. A relative index of illness was determined on a scale of 0 to 10 on the basis of severity of clinical symptoms (3, 19, 20) as follows: 0, no symptoms; 2, anorexia; 4, vomiting and/or diarrhea and/or loose stool; 6, depression and recumbency; 8, shock; 10, death. Monkeys in the stage of shock were generally fatally ill (3, 19, 20); they were euthanized.

Flow cytometry. Blood samples (0.1 ml) obtained from the monkeys were immediately stained with fluorescein- or phycoerythrin-conjugated monoclonal antibodies to leukocyte, monocyte, lymphocyte, and NK cell surface markers. Cells were stained simultaneously with two antibodies bearing contrasting fluorochromes. Erythrocytes were lysed after the staining. Cells were fixed in 1% formalin and analyzed the next day in a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems), using the PAINT-A-GATE or LYSIS II program (Becton Dickinson). Polymorphonuclear leukocytes (PMNs), monocytes, and lymphocytes were identified by light scatter and by fluorescent staining with the corresponding monoclonal antibody reagents. Ten thousand cells were analyzed for each test. When stained with anti-CD8 monoclonal antibody conjugated with phycoerythrin,

TABLE 1. Prechallenge antibody levels and illness index in monkeys challenged with aerosolized SEB

Monkey	Immunization[a]	Anti-SEB antibodies (U/100 μl of plasma sample)			Illness index[b] at day:			Outcome[c]
		IgG	IgM	IgA	1	2	3	
1	IM/IM/IM	41,670	325	10,500	0	0	0	S
2	IM/IM/IM	50,000	200	9,200	0	0	0	S
3	IM/IM	2,500	720	1,700	4	0	0	S
4	OR/OR/OR	27	60	440	4	4	6	S
5	OR/OR	10	10	120	0	0	10	D
6	OR/OR/OR	<10	50	18	4	4	10	D
7	Control	<10	44	11	4	4	10	D
8	Control	<10	25	15	4	4	10	D
9	Control	<10	12	120	0	6	10	D

[a] IM/IM/IM and OR/OR/OR = three doses of SEB toxoid; IM/IM and OR/OR = two doses of SEB toxoid microspheres. IM, intramuscular injection; OR, oral immunization by gastric intubation. See Materials and Methods for immunization and SEB aerosol challenge.
[b] O, no symptom; 2, anorexia; 4, vomiting or diarrhea or loose stool; 6, depression and recumbency; 8, shock; 10, death.
[c] S, animal survived; D, animal died.

the CD8(+) cells showed two populations: a distinct population with very bright fluorescence and a broad population with less bright fluorescence. The former cell population was defined as CD8-Hi cells, and the latter was defined as CD8-Lo cells. A similar staining pattern was seen for HLA-DR(+) cells, and the two population of cells were defined accordingly as HLA-DR-Hi cells and HLA-DR-Lo cells.

ELISA. An ELISA for quantifying monkey antibodies against SEB was performed by a modification of the procedure reported previously (4, 15). Briefly, each well of a 96-well Immulon II plate (Dynatech Laboratories, Inc., Chantilly, Va.) was coated overnight at room temperature with 75 μl of a 3-μg/ml portion of SEB in 0.1 M bicarbonate buffer (pH 9.0). Each well was then blocked with 150 μl of 1% bovine serum albumin in phosphate-buffered saline (PBS). Monkey plasma samples in serial fourfold dilutions (100 μl) were then added. Rabbit anti-monkey serum specific for the IgA, IgG, or IgM isotype in a pretitrated dilution (100 μl) was used to determine and quantify the immunoglobulin isotype. The rabbit antisera were diluted in bovine serum albumin-PBS containing 50 μg of SEB per ml and were allowed to react for at least 1 h to inhibit the residual anti-SEB antibodies that could not be removed by SEB affinity column chromatography. Peroxidase-conjugated goat anti-rabbit immunoglobulins at a pretitrated maximal dilution (100 μl) was then added. The plates were incubated for 1 h at room temperature at each step and were washed five times with PBS containing 0.05% Tween 20 (polyoxyethylenesorbitan monolaurate). Color was then developed by adding a solution (100 μl) of hydrogen peroxide mixed with ABTS [2,2'-azino-di-(3-ethylbenzthiazoline sulfonate); Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.]. The optical density was then read at a wavelength of 405 nm, with subtraction of background read at 630 nm in a Bio-Tek ELISA Kinetics Reader (Bio-Tek Instruments, Inc., Highland Park, Vt.). A monkey from our colony produced high titers of anti-SEB antibodies (midpoint titer, 9,000 for IgG, 4,200 for IgA, and 410 for IgM); this monkey plasma was used as a reference standard and was titrated in the same plates in which unknown samples were to be quantified. Antibody units per 100 μl of plasma were calculated from the reference standard. One unit of antibody was defined as the highest dilution of the reference standard that gave an optical density halfway between the maximum and the minimum (i.e., the midpoint) in the titration curve. Titration curves of the unknown monkey samples were also constructed, and the maximal dilution that gave an optical density equal to the midpoint in the titration curve of the reference standard was determined and defined as the antibody level (units) of the unknown samples.

Statistical analysis. Student's *t* test, nonparametric measures of correlation, Spearman's rank correlation coefficient (rho), and Kendall's rank correlation coefficient (tau) (12) were calculated to analyze the data, using the Statpal program (Statpal Associates, Montpelier, Vt.) installed in an IBM AT personal computer.

RESULTS

Antibody levels and survival. To study the relationship between anti-SEB antibodies and immunity to aerosolized SEB, monkeys immunized with SEB toxoid or toxoid-containing microspheres intramuscularly or orally were challenged with aerosolized SEB, and their clinical signs and ultimate outcome were observed closely for 10 days. Three nonimmunized monkeys were used as controls. The results are summarized in Table 1. Monkeys intramuscularly immunized with SEB toxoids generally produced high levels of circulating IgG and IgA antibodies, while orally immunized monkeys did not produce appreciable amounts of circulating antibodies. After challenge with aerosolized SEB, monkeys (numbers 1 to 4) with higher levels of antibodies survived, while monkeys (numbers 5 to 6) with lower levels died, as did the control monkeys (numbers 7 to 9). High antibody levels also seemed to prevent illness; monkeys (numbers 1 and 2) with the highest levels of antibodies did not show any clinical symptoms, while those (numbers 3 and 4) with lower levels of antibodies underwent various degrees of toxicosis and survived. Monkeys (numbers 5 to 9), including the controls, with the lowest levels of antibodies generally underwent illness or toxicosis progressively and died at day 3. Sickness generally began and continued intermittently with episodes of vomiting and diarrhea. Monkeys (numbers 3 and 4) were sick 2 to 36 h postchallenge but started to show no symptoms at days 2 to 4, and complete recovery was evident at days 5 to 6. These results suggest that circulating antibody levels in monkeys are critically important in immunity to SEB toxicosis and death.

Changes in antibody levels after SEB challenge. To further study the role of circulating anti-SEB antibodies, plasma samples were obtained from the monkeys (surviving and dying) before and shortly after SEB challenge, and anti-SEB

TABLE 2. Antibody levels in surviving and dying monkeys before and shortly after challenge with aerosolized SEB

Animal group	Time[a]	Anti-SEB antibodies (U/100 μl of plasma sample)[b]		
		IgG	IgM	IgA
Surviving monkeys	PRE	33,510 ± 12,990 (100)[c]	366 ± 142 (100)	6,608 ± 2,561 (100)
	T20	2,687 ± 1,024 (8.0)	133 ± 52 (36.3)	3,277 ± 1,270 (49.6)
	T90	6,433 ± 2,493 (19.2)	276 ± 107 (75.4)	3,164 ± 1,226 (47.9)
Dying monkeys	PRE	3.6 ± 1.4	52 ± 20	38 ± 15
	T20	1.0 ± 0.4	85 ± 33	25 ± 9
	T90	4.6 ± 1.8	204 ± 79	27 ± 11

[a] PRE, prechallenge; T20, 20 min postchallenge; T90, 90 min postchallenge.
[b] Values are medians at 99% confidence level ± standard error.
[c] Values in parentheses are percentages relative to prechallenge antibody levels.

antibodies were titrated. The results are summarized in Table 2. Compared with the prechallenge antibody levels, anti-SEB antibodies (IgG, IgM, and IgA) in the surviving monkeys showed a dramatic reduction at 20 min after challenge. At 90 min after SEB challenge, while IgA anti-SEB levels remained low, IgG and IgM anti-SEB antibodies showed a rebound. Comparing the immunoglobulin isotypes in the surviving monkeys, IgG antibodies showed the highest reduction (100 to approximately 8%), while IgA showed the lowest (100 to 49.6%), suggesting a difference in antibody affinity among the antibody isotypes. At all prechallenge and postchallenge times, the surviving monkeys showed significantly higher levels of anti-SEB antibodies than the dying monkeys. These results suggest that aerosolized SEB enters the blood circulation and quickly reacts with antibodies. The IgG antibodies with the highest affinity are presumably the ones consumed most quickly.

Circulating lymphocyte and leukocyte populations. To study cellular responses of the hosts in the circulation, blood samples from monkeys shortly after SEB challenge were doubly stained with monoclonal antibodies against various lymphocyte, monocyte, and leukocyte surface markers and analyzed by flow cytometry. The results are summarized in Table 3. Among the cell populations that were satisfactorily stained and characterized in the FACScan flow cytometer, both surviving and dying monkeys showed, at 20 min after SEB challenge, essentially the same proportion of PMNs, monocytes, and lymphocytes with HLA-DR, CD16 and CD56, T11 (CD2), CD8, and IL2R (CD25) markers. In contrast, at 90 min after SEB challenge, both groups of monkeys (surviving and dying) tended to show an increase in PMNs and a decrease in monocytes as well as lymphocyte subpopulations. However, these changes (from 20 to 90 min) in the surviving monkeys were not statistically significant; the changes in the dying monkeys were. Comparing the surviving and dying monkeys, there were differences in PMNs, monocytes, and various lymphocyte subpopulations; the only statistically significant differences, however, were an increase in PMNs and a decrease in cells with HLA-DR-Hi or IL2R (CD25) markers as well as in the mixed population of monocytes and NK cells with CD16 and CD56 markers. These results suggest that changes in leukocytes, monocytes, NK cells, and lymphocytes in the circulation of dying monkeys are indications of host responses to severe SEB intoxication, which are significantly expressed in the blood later than 20 min after SEB challenge. These results together with the results of antibody changes (Table 2) also suggest that anti-SEB antibodies in the surviving monkeys, within the first 20 min, efficiently prevent the aerosolized SEB from initiating the process of toxicosis and subsequent death.

Correlations among leukocyte and lymphocyte subpopulations. The changing appearance of various leukocyte and lymphocyte subpopulations immediately (20 to 90 minutes) after SEB challenge reflects the initial phase of the host response to SEB intoxication. In an attempt to understand more about this complicated cellular response, a study of the relationship among the appearance of PMNs, that of monocytes, and that of different lymphocyte subpopulations was performed by nonparametric tests of correlation. Spearman's rho and Kendall's tau were calculated, and the significance of the associations was determined. Correlation

TABLE 3. Circulating lymphocyte and leukocyte subpopulations in monkeys challenged with aerosolized SEB

Cell subpopulation	% Positive cells after SEB challenge (mean ± SD)				*P* value[a]
	20 min		90 min		
	Surviving monkeys	Dying monkeys	Surviving monkeys	Dying monkeys	
PMN	59.2 ± 4.9	59.6 ± 8.8	66.4 ± 6.2	80.8 ± 8.1	0.0216**
HLA-DR-Hi[b]	16.6 ± 1.6	17.7 ± 7.9	14.9 ± 5.8	7.1 ± 4.0	0.0486**
HLA-DR-Lo[b]	4.4 ± 1.6	5.8 ± 4.4	2.3 ± 1.6	0.7 ± 0.6	0.09
CD16+CD56	3.0 ± 0.9	3.5 ± 0.9	1.7 ± 0.3	0.6 ± 0.5	0.0094**
T11 (CD2)	17.8 ± 5.2	15.7 ± 2.8	14.8 ± 5.9	7.2 ± 4.8	0.0696
CD8-Hi	7.2 ± 2.2	8.1 ± 3.4	6.6 ± 2.8	3.7 ± 2.5	0.1527
CD8-Lo	10.4 ± 4.0	9.6 ± 4.3	9.6 ± 5.6	5.1 ± 2.9	0.194
IL2R	2.6 ± 0.9	1.5 ± 0.9	2.2 ± 1.1	0.7 ± 0.8	0.0486**

[a] *P* values are from two-tailed Student's *t* test. **, differences between surviving and dying monkeys are statistically significant.
[b] Hi and Lo are populations with high or low fluorescence stain, respectively.

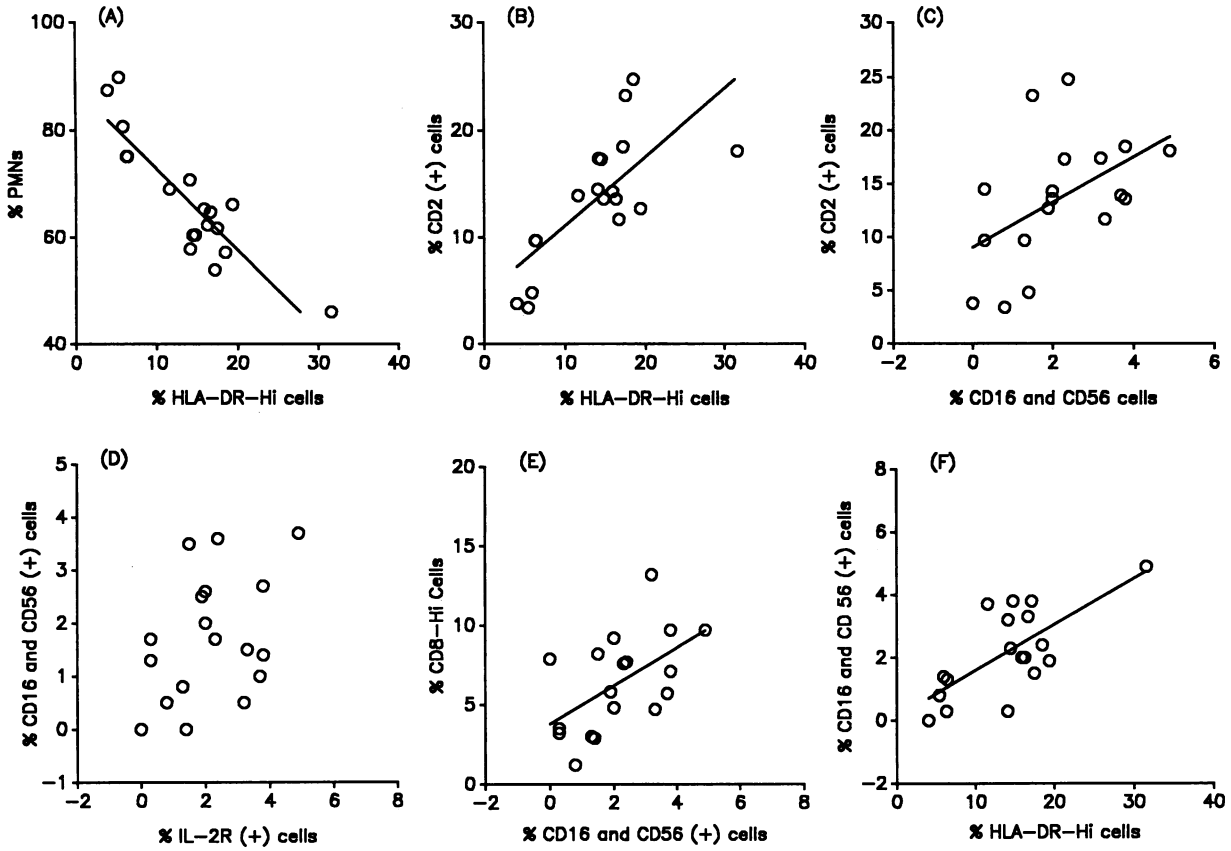

FIG. 1. Linear regression plots for correlation of the appearance of PMNs, NK cells, monocytes, and some lymphocyte subpopulations. (A) PMNs versus HLA-DR-Hi cells; tau = −0.6164; $P = 0.0002$. (B) CD2(+) cells versus HLA-DR-Hi cells; tau = 0.5461; $P = 0.0008$. (C) CD2(+) cells versus CD16 and CD56(+) cells; tau = 0.4253; $P = 0.0075$. (D) CD16 and CD56(+) cells versus IL-2R(+) cells; tau = 0.2733; $P = 0.0594$. (E) CD8-Hi cells versus CD16 and CD56(+) cells; tau = 0.3907; $P = 0.0125$. (F) CD16 and CD56(+) cells versus HLA-DR-Hi cells; tau = 0.4357; $P = 0.0061$. Hi, cell populations with high fluorescence by FACScan flow cytometry.

analyses using Spearman's rho and Kendall's tau resulted in the same conclusion. Representative linear regression plots are shown in Fig. 1. The PMNs showed an inverse correlation with all lymphocyte subpopulations as well as with the mixed population of monocytes and NK cells with CD16 or CD56 markers (exemplified by Fig. 1A). Among the lymphocyte subpopulations tested, a direct correlation was seen between any two subpopulations of lymphocytes (exemplified by Fig. 1B, C, E, and F). However, the IL-2R(+) cells did not show a significant correlation with monocytes and NK cells, which were represented by the population with CD16 or CD56 markers (Fig. 1D), suggesting that these two cellular events occurred independently. All of these results of statistical correlation analyses suggest that the initial phase of toxicosis is a complicated process that involves direct and indirect interaction of leukocytes, monocytes, NK cells, and various lymphocyte subpopulations.

DISCUSSION

We have shown here that monkeys can be immunized with SEB toxoid and SEB toxoid-containing microspheres against SEB aerosol and that circulating antibodies are a critical factor in protection from SEB aerosol challenge. A large amount of circulating antibodies (Table 1) appears to be required for protection. Monkeys without enough antibodies underwent 2 to 3 days of toxicosis and died. The dying monkeys showed a dramatic increase of PMNs and decreases of monocytes, NK cells, and various lymphocyte subpopulations in the circulation shortly after SEB challenge. The decrease in the antibody levels in the surviving monkeys and the dramatic changes of PMNs, monocytes, NK cells, and lymphocyte subpopulations seem to occur sequentially, suggesting that anti-SEB antibodies function before these cellular responses occur. The changes in leukocytes, monocytes, NK cells, and lymphocyte subpopulations may be an indication of initial toxicosis, which seems to involve complicated cellular interactions.

The present study shows that large amounts of anti-SEB antibodies in the circulation correlated with protection against lethal SEB aerosol challenge in surviving monkeys. Although the characteristics of antibodies were not defined, a similar conclusion was reached in classical studies in which monkeys with high levels of circulating anti-SEB antibodies were immune or resistant to intravenous and possibly aerosolized challenges with SEB (5, 6, 28, 40). In the present study, monkeys intramuscularly immunized with three doses of toxoid produced a relatively high level of antibodies, and they survived the aerosol challenge without any signs of sickness. In contrast, monkeys with lower levels of antibodies were sick temporarily and survived. Almost immediately (20 min) after SEB challenge, the level of anti-SEB antibodies decreased dramatically. However, IgG and IgM antibodies rebounded quickly; at 90 min after challenge, an appre-

ciable increase of antibody levels was seen. During this time of recovery in the surviving monkeys, leukocytes, monocytes, NK cells, and various lymphocyte subpopulations changed significantly in the dying monkeys. Thus, there seems to be a time linkage between the consumption of antibodies and changes of circulating leukocytes, monocytes, and lymphocytes. The critical time that decides survival and death seems to be in the first 20 min after SEB challenge.

The lungs are paired organs that are extended from the trachea via two primary bronchi and function to aerate the blood (46). The airway network in the lung is similar to the pattern of a tree; it starts with a bronchus, which branches out progressively into bronchioles, which then progressively end with numerous alveoli. The submucosa becomes thinner and thinner from the bronchi to the bronchioles and disappears in alveoli. The alveolus is essentially a single cell layer surrounded by a network of capillary blood vessels. Numerous alveoli form a huge interface network that allows a direct and efficient exchange of air in the atmosphere with the blood in the circulation. It has been suggested that secretory IgA in the bronchi and upper bronchioles and circulating antibodies, particularly IgG, in the lower bronchioles and alveoli are the first lines of defense against infectious agents and noxious substances (7). Because of the unique structure of the alveoli, it is not surprising to see in the present study that a large amount of SEB reaches the blood circulation immediately after aerosol challenge. The SEB that enters the alveoli is quickly neutralized by the circulating antibodies in the surviving monkeys, while SEB not neutralized by the antibodies in the nonsurvivors enters the blood circulation, reaches various organs, and causes toxicosis, toxic shock, and death. The lungs are probably the organs that are most affected by the SEB aerosol, as postmortem pathological examinations revealed a general pattern of severe edema with perivascular infiltrations of neutrophils and monocytes. Although the pulmonary edema and dysfunction have been suggested to be the cause of death in SEB toxemia (24), it is more likely in the present study that death from SEB aerosol-induced toxic shock is the combined result of functional failure and pathological changes in several systems, organs, and tissues (19a).

Although we did not measure anti-SEB antibodies in the lungs because of technical difficulties in obtaining satisfactory bronchoalveolar lavage samples, secretory antibodies in the lungs may also play an important role in the first line of defense against aerosolized SEB in our monkeys (7). However, these mucosal antibodies seem to have been overwhelmed by the large amount of SEB that reached the lungs and entered the blood circulation. This entrance of SEB into the circulation could be due to an insufficient amount of anti-SEB antibodies present at the time of aerosol challenge. Monkeys that survived without any clinical symptoms, although they had large amounts of antibodies in the circulation, seem not to have had sufficient antibodies in the lungs either, as a dramatic decrease of anti-SEB antibodies was seen in these monkeys.

The respiratory and gastrointestinal (GI) tracts are parts of the common mucosal immune system (7, 27, 29, 45). Immunization of the GI tract generally results in the production of IgA antibodies, not only in the GI tract but also in the lung mucosa; conversely, immunization of the lungs often results in the production of antibodies in both the lungs and the GI tract (7, 27, 29, 30, 32, 45). The antigens that reach the mucosal surface are taken up by a specialized type of epithelial cells termed M cells or follicle-associated epithelial cells (8, 33), which cover the Peyer's patches or bronchus-associated lymphoid tissue and are transported to the lymphoid elements, where commitment to IgA production occurs (7, 9, 13, 42). It is generally agreed that mucosal immunity plays a major role in protecting hosts from insults by mucosal infectious diseases and noxious substances (reviewed in references 7 and 30). However, in our study, oral immunization with SEB toxoid or SEB toxoid-containing microspheres generally did not result in immunity to a lethal challenge with aerosolized SEB, and the circulating antibody levels were relatively low. The reason for this failure to produce high levels of anti-SEB antibodies with oral immunization is unknown. It could be because mucosal immunity is generally short-lasting and that, in consequence, antibody levels are low at the time of SEB challenge, or that SEB is not efficiently taken up by the mucosal cells, particularly the M cells in the GI tract, to generate long-lasting immunity, or that there is oral tolerance in the GI tract (2, 30, 41) due to exposure to food contaminated with minute quantities of staphylococcal enterotoxins, including SEB. Oral immunization to induce mucosal antibodies has historically been difficult due to a variety of technical obstacles (18, 35).

Although secretory IgA antibodies are generally produced in mucosal tissues, essentially all circulating IgA antibodies in the blood are produced in the bone marrow (30, 31). The vast majority of these circulating IgA antibodies, however, are not transported to mucosal surfaces (30, 31). Surprisingly, this compartment of IgA antibody responses has been neglected in mucosal immunity research. Nevertheless, in the present study, even though we failed to induce large amounts of mucosal antibodies, it is not surprising to see substantially high levels of IgA antibodies in the circulation of surviving monkeys that had been immunized intramuscularly. These IgA antibodies may well have been produced in the bone marrow and released into the circulation. The function of these IgA antibodies, besides neutralizing the SEB that entered the circulation, is unknown. It has been suggested that circulating IgA antibodies do not activate complement and thus may compete with IgM and IgG antibodies in binding antigens that otherwise would have activated complement-mediated effector mechanisms (17). The reason that intramuscularly immunized monkeys with high IgG antibody levels survived SEB challenges without any anaphylactic reactions could be due to the presence of high levels of IgA antibodies in the circulation that reduced the chance of activating the complement system by IgG and IgM antibodies.

In surviving monkeys, due to the effects of circulating antibodies, there were insignificant changes in PMNs, monocytes, and lymphocytes of various populations shortly after SEB challenge. However, in dying monkeys 90 min after SEB challenge, there was a dramatic increase of PMNs and a dramatic decrease of NK cells and monocytes (CD16 and CD56 markers) and of lymphocytes with HLA-DR, CD2, CD8, or IL2R surface markers. If SEB toxicosis and death are initiated by the interaction of antigen-presenting cells, in the context of class II antigens, with T cells bearing certain types of T-cell receptors (11, 21, 22, 26), the disappearance of some of the HLA-DR(+) cells, monocytes, and T cells seems to be consistent with this possibility. These disappearing cells may localize in tissues, interact with each other, and initiate the cascade or combination of pathophysiological changes that results in toxicosis and death. However, the initial phase of SEB toxicosis seems to be more complicated; we have also seen in dying monkeys a significant decrease of

NK and IL-2R(+) cells which are not significantly correlated with each other and may also play important and independent roles in the process of toxicosis and death. Further studies on the roles of all of these cell populations in later phases of toxicosis coupled with studies of mediators, clinical symptoms, and postmortem histopathology should provide valuable information about the mechanism of SEB toxicosis and death.

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