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In Vivo Effect of Staphylococcal Enterotoxin A on Peripheral Blood Lymphocytes

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Staphylococcal enterotoxin A (SEA) administration to monkeys produced an initial lymphocytic leukopenia lasting approximately 24 h. Lymphocytes isolated from blood circulation (PBL) during this stage had normal or decreased [^{3}H]thymidine incorporating activity. After 48 h, however, a significant increase (five- to sixfold) in [^{3}H]thymidine incorporating activity into PBL was apparent. The peak of incorporating activity (seven- to eightfold) was reached 3 to 4 days after SEA administration, followed by a gradual decline, reaching the baseline after 2 weeks. The increased levels of [^{3}H]thymidine incorporation in PBL were concomitant with the conversion of lymphopenia into lymphocytosis, accompanied by the release of many immature cells into the circulation. Lymphocytes isolated 24 h after SEA administration in vivo did not respond to the mitogenic action of SEA in vitro. Lymphocytes isolated at later stages after SEA challenge were fully activated by toxin. From a series of studies, it was concluded that SEA administered to monkeys caused, during the initial 24 h, the removal of a great proportion of lymphocytes from the circulation, followed by the release of new immature cells with augmented DNA synthesis activity. The lymphocytic leukocytosis state declined gradually and reached normal levels between 3 and 4 weeks after the SEA challenge. The biological implications of the hematological changes occurring after SEA challenge in vivo are discussed.

Staphylococcal enterotoxins are a family of structurally related proteins which, in minute quantities, cause nearly half of the food poisonings in humans (2). Experimental enterotoxemia with the main symptoms of emesis and diarrhea can be produced in monkeys or cats by intravenous or oral administration of these toxins (1, 2). Among other symptoms associated with the experimental toxemia is a transient leukopenia followed by a neutrophilic leukocytosis which subsides to normal after 28 h (4, 18). Another main biological characteristic of staphylococcal enterotoxins is their high potency in vitro as mitogens for mouse and human T lymphocytes (9, 10, 16). It has also been shown that staphylococcal enterotoxin A (SEA) is better than concanavalin A (ConA) or phytohemagglutinin P in inducing immune interferons (interferon gamma) and suppressing the primary plaque-forming cell response of human peripheral blood lymphocytes and mouse spleen cells (8, 9, 11). The purpose of the present study was to examine the possibility that lymphocyte activation by a staphylococcal toxin takes place in vivo.

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MATERIALS AND METHODS

Monkeys. Baboon monkeys, weighing 3.5 to 5 kg, were used. All monkeys were screened to ensure the absence in their sera of antibodies against SEA and staphylococcal enterotoxin B (SEB). The enterotoxins were administered into the femoral vein, and blood samples were obtained immediately before and at selected times after challenge. Leukocytes were counted by the usual hemacytometer procedures on blood obtained from the femoral vein. Differential counting was done in blood films prepared with Giemsa stain.

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Staphylococcal enterotoxins, toxoid, and other lectins. Purified SEA was prepared as described by Schantz et al. (14) and kindly supplied by the late M. Komarov, Health Department, Haifa, Israel. SEB was purchased from Makor Chemicals Ltd., Jerusalem, Israel. Both toxins were further purified by chromatography on hydroxylapatite (Bio-Gel HT; Bio-Rad Laboratories, Richmond, Calif.) by published methods (14). The final products ran as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. No traces of hemolytic activity due to α and β hemolysins were detected in purified SEA and SEB by available techniques (19). SEB toxoid was prepared from purified toxin by formaldehyde treatment as described by Silverman et al. (15). ConA, phytohemagglutinin type V (PHA), pokeweed mitogen (PW), and lipopolysaccharide from *Escherichia coli* were purchased from Sigma Chemical Co., St. Louis, Mo.

Peripheral blood lymphocyte suspensions. Blood (4 to 5 ml) was collected from monkeys in the presence of heparin (100 USP units). The blood was diluted with an equal volume of phosphate buffered-saline (PBS), and lymphocytes were separated by gradient centrifugation on Ficoll-Paque (3). The gradient-separated lymphocytes were washed three times in PBS and suspended in RPMI 1640 medium (2×10^6 lymphocytes per ml) containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer and supplemented with 5% fetal bovine serum and antibiotics (100 U of penicillin per ml and 100 μg of streptomycin per ml).

[^{3}H]thymidine incorporation into lymphocytes. The [^{3}H]thymidine incorporation capacity of lymphocytes was measured in triplicate samples of 150 μl (2×10^5 cells), cultured in a micro tissue culture plate (Sterilin micro test plate; flat bottom). [^{3}H]thimidine (1 μCi; [*methyl*-1', 2'-^{3}H]thymidine; 128 Ci/mmol; Amersham) in 10 μl of RPMI medium was added to each well at the beginning of incubation. Incubation was conducted for 24 h at 37°C in a humidified CO_2-air incubator, and the incorporation was stopped by pipetting aliquots (100 μl) of each sample on 3MM Whatman paper disks (25-mm diameter). The disks

TABLE 1. [^{3}H]thymidine incorporation into peripheral blood lymphocytes of monkeys exposed to SEA, SEB, and SEB toxoid

Days after treatment	cpm of [^{3}H]thymidine incorporated per 0.1 ml of lymphocytes isolated from monkeys treated with[a]:			
	Saline (n = 4)	SEA (n = 12)	SEB (n = 4)	SEB toxoid (n = 4)
0	970 ± 293	818 ± 254	712 ± 131	1,128 ± 261
1	952 ± 209	760 ± 454	1,084 ± 404	1,377 ± 405
2	1,296 ± 414	4,203 ± 1,912	5,059 ± 1,118	1,221 ± 188
3		6,276 ± 2,373		
4	961 ± 174	6,494 ± 2,795	6,211 ± 4,203	1,227 ± 353
8	836 ± 205	1,865 ± 724	831 ± 310	1,172 ± 146
11			490 ± 184	
16	849 ± 120	915 ± 698		

[a] Results are expressed as the mean ± standard deviation of values from the number (n) of monkeys treated with enterotoxin, toxoid, or saline.

were left for 20 min at room temperature, washed three times with 5% trichloroacetic acid and once with 75% ethyl alcohol–25% ethyl ether, and dried in a 50°C oven. Finally, the disks were inserted into vials containing a liquid scintillation fluid (Packard Toluene Scintillator: POPOP [1,4-bis-(5-phenyloxazolyl)benzene], 0.1 g/liter; PPO [2,5-diphenyloxazole], 5 g/liter) and counted in a Packard liquid scintillation counter.

Determination of mitogenic activity in vitro. Lymphocytes isolated from control monkeys and monkeys challenged with staphylococcal enterotoxin were incubated in the presence and absence of SEA as indicated above. After 48 h of incubation, 1 μCi of [^{3}H]thymidine was added to each well, and the incubation was continued for an additional 6 h. Processing and determination of [^{3}H]thymidine incorporation were done as described above.

Determination of peripheral blood lymphocyte life span. Lymphocytes were isolated from 8 ml of blood essentially by the same method as described above. To avoid erythrocyte or other cell contamination the lymphocytes were passed twice through a Ficoll-Paque gradient. The washed lymphocytes were suspended in 0.3 ml of PBS containing 300 μCi of ^{51}Cr-labeled sodium chromate (300 to 500 μCi/mg of Cr; Nuclear Research Center, Negev, Israel) and incubated at 37°C for 1 h. The cells were washed once in 10 ml of RPMI 1640 medium, resuspended in the same medium, and incubated for 1 h at 37°C. The last wash was performed with PBS, and the lymphocytes were resuspended in 2 ml of PBS and reinjected intravenously into the same monkeys. Blood samples (1 ml each) were collected daily and counted in a Beckman gamma counter. The first blood sample was collected after 16 h to ensure the removal of surface-adhered label.

RESULTS

[^{3}H]thymidine incorporation into lymphocytes isolated from staphylococcal enterotoxin-treated monkeys. Monkeys were challenged intravenously with SEA (0.5 μg/kg of body weight). Blood samples were removed immediately before toxin administration and at various times after treatment. Lymphocytes isolated from the various blood samples were tested for [^{3}H]thymidine incorporation activity. During the first 24 h after toxin administration, there was a decrease in [^{3}H]thymidine incorporation in the lymphocytes in some of the experiments, whereas in other experiments there was no significant change in comparison to zero-time lymphocytes (Table 1). At 48 h after SEA administration there was a remarkable enhancement of DNA synthesis activity in lymphocytes as measured by their [^{3}H]thymidine incorporating activity. In most experiments (10 of 12) the maximal activity of the lymphocytes was noticed at days 3 and 4, followed by a gradual decline, reaching the control level only 2 weeks after toxin administration. Monkeys treated with SEB (0.5 μg/kg of body weight) reacted to this toxin essentially in the same way as to SEA, whereas exposure of monkeys to SEB toxoid (1 μg/kg of body weight) did not induce any significant reaction that differed from the control (Table 1). Experiments were also repeated with lower doses of SEA to establish the minimal toxin dose still affecting the [^{3}H]thymidine incorporation into peripheral blood lymphocytes. The administration of 0.1 μg of SEA per kg of body weight produced basically the same effects as those described in Table 1. Lymphocytes of monkeys challenged with 0.02 μg of SEA per kg of body weight showed a twofold increase in [^{3}H]thymidine incorporating activity at days 2, 3, and 4, subsiding to normal at day 5 (results not shown). In some of the control monkeys, mere bleedings caused a slight enhancement (much lower than in SEA or SEB experiments) of their lymphocyte incorporating activity at day 2 or 3 (Table 1). The general leukocyte response in monkeys to staphylococcal enterotoxin challenge was studied concomitantly with the DNA synthesis activity of the lymphocytes. At 24 h after SEA administration an insignificant decrease in the total number of leukocytes was observed (Fig. 1). However, upon differential counting of those blood samples, a change toward a neutrophile-predominant population was observed. The peak of leukopenia was reached after 48 h. At that point, however, the ratio of granulocytes to lymphocytes was reversed. From day 2 onward, leukopenia converted into a mainly lymphocytic leukocytosis lasting for nearly 2 weeks. Normal leukocyte levels were observed after 3 to 4 weeks. The characteristic of the leukocytosis occurring between days 2 and 7 was the presence of a large proportion of immature lymphoid cells (large lumphocytes with basophilic cytoplasm) (Fig. 1). The abundance of a great proportion of immature cells among the leukocytes explains the increase in the DNA synthesis capacity of lymphocytes isolated from monkeys during the first week after treatment.

In vitro response of lymphocytes from SEA-challenged monkeys to mitogenic induction. Lymphocytes isolated from monkeys before toxin administration and at various times afterward were tested for their capability to respond in vitro to SEA. Lymphocytes isolated from monkeys 24 h after SEA challenge did not respond, fully or partially, to SEA in vitro (Fig. 2). Those lymphocytes reacted also less efficiently to ConA, PHA, and PW (a T-lymphocyte-dependent B-lymphocyte mitogen) (Fig. 2). We also tested the stimulation capacity of lipopolysaccharide from *E. coli* in normal lym-

phocytes and lymphocytes isolated at various times after SEA treatment, but neither of those lymphocytes was induced significantly by this B-lymphocyte mitogen (results not shown). The full or partial loss of reactivity of lymphocytes from monkeys 24 h after SEA challenge toward the mitogens listed above was, however, a transient phenomenon. Lymphocytes isolated after longer periods after SEA administration retained their ability to react to mitogenic stimuli (Fig. 2).

The transient loss of response to mitogens of lymphocytes from monkeys after SEA treatment could be caused by the following. (i) Those lymphocytes, although still in circulation, lost the ability to respond to mitogens or their response was inhibited by suppressor cells. (ii) The lymphocytes that were in contact with SEA were recruited transiently from circulation. The following experiments were carried out to examine the aforementioned possibilities:

(i) The ability of lymphocytes to respond in vitro to SEA after a short exposure to the same toxin was examined. Lymphocytes were isolated from control monkeys and incubated for 1 h with SEA, followed by thorough washing of the cells with PBS. One hour was chosen as the incubation time, since most of the enterotoxin administered to monkeys is removed from the plasma during the first hour (4). The washed lymphocytes were resuspended in fresh medium, and their [^{3}H]thymidine incorporation was tested in the presence and absence of SEA immediately, after 24 h, and after 48 h of incubation. Exposure of lymphocytes for only 1 h to SEA, followed by the removal of SEA from the medium, was sufficient to induce full mitogenic activity in those cells. No addition of new mitogen was needed for the full expression of lymphocyte activation after 48 h and partial activation after 24 h, suggesting a tight binding of SEA to lymphocytes (Table 2). Based on in vitro experiments, one would assume that increased [^{3}H]thymidine incorporating activity would also be found in in vivo experiments after SEA administration. This, however, was not the case, probably due to suppressor cells or removal of SEA-affected lymphocytes from circulation.

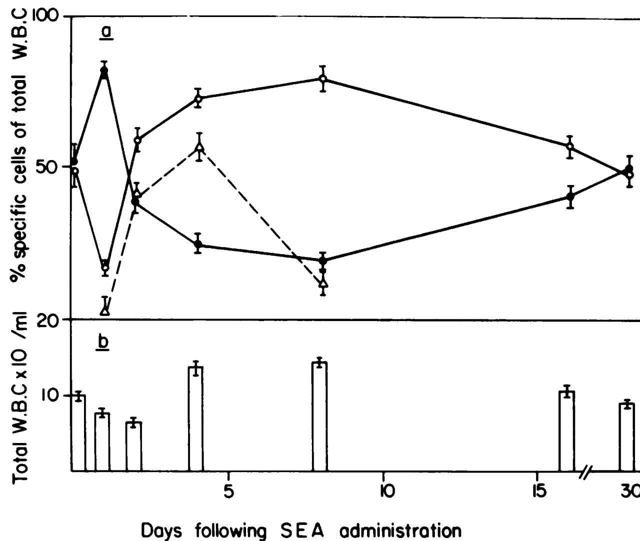

FIG. 1. Leukocyte (W.B.C.) population after SEA administration to monkeys. Monkeys were injected intravenously with 0.5 μg of SEA per kg of body weight. Total leukocyte counts and differential counts were performed on heparinized whole blood taken before SEA injection and at various times afterward. (a) Changes in main leukocyte populations after SEA challenge. Symbols: ●, percent polymorphonuclear leukocytes of total leukocytes; ○, percent lymphocytes of total leukocytes; △, percent large lymphocytes as calculated from the total number of lymphocytes only. (b) Leukocyte counts after SEA challenge. Bars show standard error of the mean of values obtained from seven monkeys.

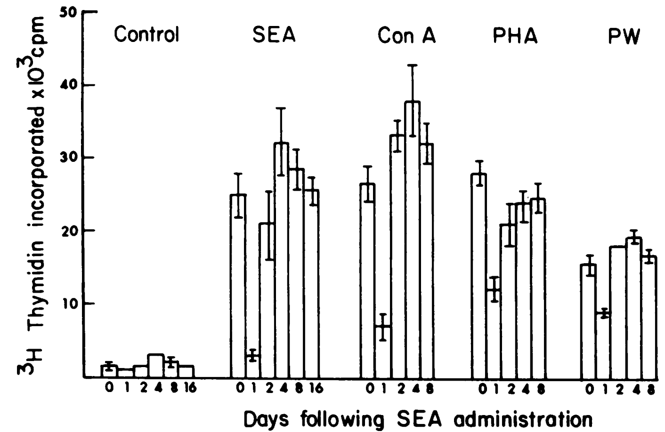

FIG. 2. Effect of various mitogens on lymphocytes from SEA-challenged monkeys. Lymphocytes isolated at various times after SEA administration were exposed in vitro to different mitogens (SEA, 20 ng/ml; ConA, 2 μg/ml; PHA, 10 μg/ml; and PW, 5 μg/ml), and their [^{3}H]thymidine incorporating capacity was determined. Standard error is not shown where the bars were smaller than symbols. Number of monkeys tested for each mitogen: SEA, 7; ConA, 4; PHA, 4; PW, 4; and saline, 7.

(ii) To verify the possibility that SEA administration in vivo induced the release of short-lived suppressor cells into the circulation or activated existing suppressor cells and those suppressor cells prevented the response of lymphocytes to the mitogenic effect of SEA and other mitogens in vitro, the following experiment was carried out. Control lymphocytes were isolated from a monkey before SEA treatment and incubated at 37°C until used. Twenty-four hours after SEA administration, a blood sample was taken, and lymphocytes were isolated. The two lymphocyte samples were tested separately and together for their response to SEA in vitro. The results clearly demonstrate that although lymphocytes, 24 h after SEA treatment, do not respond to the mitogenic action of SEA in vitro, they also do not elicit any suppressing activity on the mitogen-dependent stimulation of [^{3}H]thymidine incorporation in normal lymphocytes (Table 3).

(iii) To gain more direct evidence on the recruitment of lymphocytes from circulation of monkeys challenged with SEA, their lymphocyte life span was studied. Blood samples (8 ml each) were removed from monkeys, and lymphocytes were isolated and labeled with ^{51}Cr-labeled sodium chromate as described above. The labeled lymphocytes were injected intravenously into the original monkeys. Sixteen hours after lymphocyte transfusion, 1 ml of blood was removed, after which two monkeys were challenged with SEA and two control monkeys with saline. Daily, 1-ml blood samples were collected from the monkeys, and, after a follow-up of 12 days, all the blood samples were counted in a Beckman gamma counter. It appears (Fig. 3) that normal monkey lymphocytes consist of two main populations, as derived from their life span. One population has a half-life of nearly 3 days, and the second has a half-life of 5.5 days. Administration of SEA into the blood circulation shortened drastically

TABLE 2. Mitogenic induction of SEA-pretreated lymphocytes for various times[a]

Pretreatment of lymphocytes	Mitogen added (μg/ml)	[³H]thymidine incorporation[b] (cpm/0.1 ml of cell suspension)		
		0 h	24 h	48 h
None		1,260 ± 145	980 ± 85	1,130 ± 75
None	0.02 SEA	1,410 ± 65	9,635 ± 910	14,630 ± 1,915
None	2 ConA	1,340 ± 90	12,640 ± 895	23,615 ± 2,015
1 h of incubation with SEA		1,315 ± 105	8,960 ± 1,200	22,000 ± 2,160
1 h of incubation with SEA	0.02 SEA	1,475 ± 110	11,020 ± 780	25,780 ± 1,700
1 h of incubation with SEA	2 ConA	1,530 ± 115	17,630 ± 1,435	32,645 ± 2,945

[a] Two 5-ml lymphocyte cell suspensions (2×10^6 cells per ml) were incubated for 1 h with 20 ng of SEA per ml and without SEA. Unbound SEA was removed from cells by three successive washes with PBS. Each cell suspension was resuspended in fresh medium and incubated for various periods of time as indicated, followed by a 4-h incubation with [³H]thymidine.

[b] Mean of triplicate samples ± standard deviation.

the life span of both populations to half-lives of less than 1 day and 4.2 days. This experiment showed unequivocally the direct effect of SEA administration on the partial removal of lymphocytes from circulation.

DISCUSSION

The results of the present experiments, along with those reported and described by others (4, 18), show that administration of minute amounts of staphylococcal enterotoxin (SEA or SEB) into monkeys induces an initial state of lymphopenia which lasts for 1 to 2 days. Our extended studies on the lymphocytes isolated during this stage showed that the [³H]thymidine incorporating capacity of those cells was low, although not significantly different from control lymphocytes. The responsiveness of those lymphocytes toward SEA in vitro was, however, fully or partially depressed, depending on the dose and kind of toxin administered. By day 2 after staphylococcal enterotoxin treatment, the granulocyte to lymphocyte ratio was reversed, followed by total increase in lymphocytes, peaking between days 3 and 7. Lymphocytosis was associated with the emergence of immature cells into circulation concomitant with greatly enhanced [³H]thymidine incorporation of these lymphocytes. The increased activity of lymphocytes, together with the general lymphocytosis, declined gradually, reaching control levels between 2 and 3 weeks after SEA challenge. The transient refractory state of lymphocytes to SEA and other T-cell-dependent mitogens in vitro after toxin administration to monkeys could be explained in several ways, and experiments were conducted to select among them. Studies in vitro, in which the effect of short SEA exposure on lymphocyte activation was measured, ruled out the possibility that SEA bound to lymphocytes in the first place would elicit the unresponsiveness of these cells to mitogens at a later stage. The refractory state of the 24-h lymphocytes toward SEA, caused by the induction of suppressor cells after SEA treatment, was also rejected, based on the results of mixing experiments in which those lymphocytes did not exert any suppressing effects on control lymphocytes if incubated together. It should, however, be stressed that suppressor cells have been observed in other experimental systems after mitogen induction (SEA, ConA, and PHA) and natural virus infections (5, 7, 12). This difference in results may be a consequence of the experimental system or the intensity of induction. The observed lymphopenia, together with the rapid removal of lymphocytes from circulation after SEA treatment, implied that full or partial unresponsiveness of the 24-h lymphocytes toward T-cell-dependent mitogens could be considered as the period during which mitogen-sensitive cells were withdrawn from circulation, in analogy to antigen-induced selective recruitment of circulating lymphocytes (6, 13, 17). Whether the recruitment of lymphocytes induced by SEA challenge is selective for certain T-cell populations or is a more general phenomenon is not clear from our data (Fig. 2). Final conclusions must await experiments involving surface marker analysis of lymphocyte populations after SEA treatment. The termination of unresponsiveness may be associated with the emergence of SEA-stimulated lymphocytes from lymphoid organs in which their activation and propagation took place. Lymphocytosis might be a consequence of nonspecific hyperplasia triggered by the SEA-dependent rapid recruitment of lymphocytes.

Emesis and diarrhea, the main symptoms of food poisoning and experimental enterotoxemia caused by staphylococcal enterotoxin, usually persist for less than 24 h (1, 2). Other clinical symptoms, as well as hematological and biochemical changes, have been observed during the first 24 h after toxin administration or ingestion (2, 4, 18). Our data describe the existence of highly activated lymphocytes (as measured by their [³H]thymidine incorporation) concomitant with dramatic hematological changes which persisted long after all clinical symptoms had disappeared. These changes, taking place after SEA challenge, may be representative of immunopotentiation (perhaps through induction of interferon gamma or other lymphokines) or suppression of the immune system and as such may have important medical implications

TABLE 3. Mitogenic response to SEA toxin of an in vitro peripheral blood lymphocyte mixed culture derived from a normal and an SEA-treated monkey[a]

Source of peripheral blood lymphocytes	[³H]thymidine incorporation[b] (cpm/0.1 ml of cell suspension)
Normal	52,510 ± 4,157
SEA treated	625 ± 73
Normal + SEA treated	31,040 ± 2,164

[a] Lymphocytes isolated from a monkey before SEA administration and incubated at 37°C for 24 h were considered normal lymphocytes. The mitogenic response to SEA was determined on 100-μl normal lymphocyte suspensions, 100-μl lymphocyte suspensions from the same monkey 24 h after SEA administration, and a mixture of 50-μl cell suspensions of each.

[b] Mean of triplicate samples.

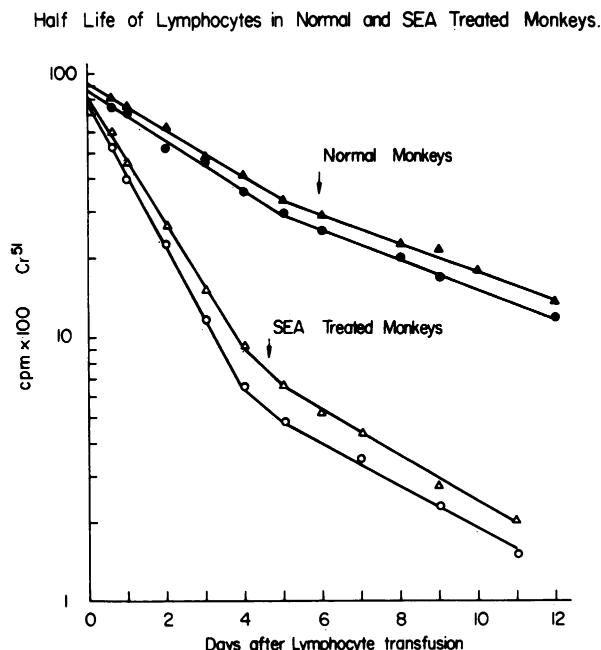

FIG. 3. Lymphocyte life span in normal and SEA-treated monkeys. Four monkeys were transfused with their own ^{51}Cr-labeled lymphocytes (5×10^7 cells per monkey; each sample contained between 2.7×10^6 and 2.1×10^6 counts). Sixteen hours after lymphocyte transfusion, two monkeys were injected with 0.5 μg of SEA per kg of body weight, and two other monkeys were injected with saline. Blood samples (1 ml each) were taken from the monkeys every 24 h during the course of 12 days.

concerning resistance or vulnerability to infection after exposure to staphylococcal enterotoxins. Studies in our laboratory are in progress to establish whether the toxin administered in vivo exerts a stimulatory or suppressing effect on the cellular immune system of monkeys.

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