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Antigen-Induced Selective Recruitment of Circulating Lymphocytes [1,2]

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Thoracic duct lymphocytes obtained from mice 1–2 days after the injection of sheep erythrocytes and injected into thymectomized, irradiated, marrow-protected syngeneic hosts were deficient in adoptively transferring immune reactivity to sheep erythrocytes but normal with respect to horse erythrocytes. Cells collected at 3 days had normal reactivity, but cells collected at 5 days allowed their hosts to produce an enhanced response to sheep erythrocytes and a somewhat depressed response to horse erythrocytes. Thoracic duct lymphocytes obtained from CBA mice 2 days after injection of CBA × C57BL)F_1 spleen cells produced splenomegaly in newborn (CBA × BALB/c)F_1 mice but not in (CBA × C57BL)F_1 recipients. When obtained 5 days after injection, they caused an increase in the splenic index of the (CBA × C57BL)F_1 recipients significantly above that given by control lymphocytes from saline-injected donors. These results are interpreted in terms of a selective recruitment from the circulation of specific antigen-sensitive cells, occurring soon after antigen administration and followed in turn by a rapid reentry of such cells into the pool in an increased proportion.

INTRODUCTION

It is now well established that small lymphocytes recirculate from blood through lymphoid tissues into lymph (1). It has been suggested that the process of recirculation may afford a physiological mechanism enabling the recruitment of immunocompetent cells into sites regionally stimulated by antigen (2, 3). The number of antigen-sensitive lymphocytes able to bind antigen specifically is a very small proportion of the total cell population (4). Large-scale recirculation of lymphocytes through lymphoid tissues, in comparatively short periods of time (5), would thus ensure that those cells capable of responding to the antigen could be recruited into the appropriate site.

Circumstantial evidence revealing the importance of lymphocyte migration in im-

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mune induction has been obtained in a variety of experiments. Thus, for instance, after local delivery of a high dose of irradiation to a single lymph node (6) or to the spleen (7), the small lymphocyte population of the organ was destroyed but rapidly replenished by blood-borne cells. Local antibody production following antigenic stimulation was thus not diminished. Furthermore, in experiments with isolated perfused rat spleen, the magnitude of the antibody response to sheep erythrocytes was proportional to the concentration of lymphocytes in the perfusate and not to the content of lymphocytes initially present within the spleen (8). The addition of sheep erythrocytes to the perfusate did not alter the rate of migration of lymphocytes into the spleen although it did cause a transient inhibition in the subsequent discharge of these cells (5). In the sheep, antigenic stimulation of the popliteal lymph node led to a virtual arrest of the emigration of lymphocytes from the node for about 6 hr (9). The functional significance of these alterations in lymphocyte traffic is not clear. Nevertheless, the concept of recruitment of lymphocytes following antigenic stimulation implies a selective withdrawal of antigen-sensitive cells from the circulation into stimulated lymphoid organs. As a corollary, it might be expected that there would be, for a short period of time thereafter, a concomitant reduction of specific antigen-sensitive cells from the population of circulating lymphocytes. The present experiments were designed to test this corollary.

MATERIALS AND METHODS

Animals. Female mice of the highly inbred CBA strain (originally obtained from Harwell, Didcot, Berkshire, England) were used throughout. They were raised and maintained at the Hall Institute and were fed Barastoc cubes with an occasional green feed supplement of cabbage and given water *ad libitum.* Neonatal F_1 hybrid mice from crosses between CBA and C57BL and between CBA and BALB/c were used in graft-versus-host assays. The C57BL and BALB/c strains were highly inbred and maintained at the Hall Institute under the same conditions as the CBA strain. They were obtained from Dr. R. Bradley of the University of Melbourne and originally derived from a stock maintained by Dr. L. W. Law of the National Institutes of Health, Bethesda, Md.

Cell suspensions. Thoracic duct lymphocytes were obtained as described below. After washing in Dulbecco's phosphate buffer (10) in the cold, the cells were resuspended in that medium, counted in a hemocytometer, and the volume adjusted to obtain the desired concentration for injection.

Bone marrow cells were expressed from the femurs and tibiae by means of a syringe and attached needle containing cold Eisen's medium (11). The marrow plugs were gently disrupted by aspiration through a 25 gauge needle and the suspension of single cells was washed once, resuspended in cold Eisen's medium, counted, and the volume adjusted to give the desired concentration.

Cell suspensions from spleen were obtained by teasing with fine forceps through an 80 mesh stainless steel sieve in cold Eisen's solution, washing twice and proceeding henceforth as for bone marrow above.

Sheep erythrocytes were obtained from a single animal by jugular vein puncture. The blood was stored in Alsever's solution for 1 week. Before use the cells were

washed 3 times in saline and finally resuspended to an appropriate volume in saline.

Horse erythrocytes were obtained from the Commonwealth Serum Laboratories, Melbourne, Australia, and stored in citrate saline. When required, they were washed 3 times in saline and resuspended in saline to an appropriate volume.

Injections. Cell suspensions were injected into the tail vein of adult mice or intraperitoneally in neonatal mice.

Operative procedures. Thoracic duct fistulas were established in 8–10-week-old mice using our modification (12) of the technique described by Boak and Woodruff (13). During the collection, the mice were restrained in modified Bollman cages (14) and the lymph allowed to drain in plastic tubes kept in ice water in vacuum flasks and containing 10% fetal calf serum in Dulbecco's medium and 50–100 IU of preservative free heparin (Evans Medical Ltd., Liverpool, England) per ml. Parenteral or oral administration of fluid was not given. Approximately 80% of the mice were alive 6 days after the operation and of these about 80% drained lymph throughout.

Thymectomy was performed in 7-week-old CBA mice according to the method of Miller (15). Examination of the mediastinal areas of thymectomized mice at the termination of the experiments revealed no thymus remnants in any of the animals.

Irradiation. Thymectomized mice were exposed to total body irradiation in a Perspex box generally 3–5 weeks after thymectomy. The dose given was 800 R to midpoint with maximum backscatter conditions and the machine operated under conditions of 250 kV, 15 mA, and an HVL of 1 mm Cu. The focal skin distance was 50 cm and the absorbed dose rate was 170 R/min. Thymectomized mice were protected with bone marrow a few hours after irradiation by injecting them with 5 million syngeneic cells. A further period of 3–5 weeks was allowed to elapse before these mice were used as recipients in adoptive transfer experiments. All irradiated mice received polymyxin B (100,000 IU/l.) and neomycin (10 mg/l.) in the drinking water.

Detection of antibody-forming cells. Spleen cell suspension for assays were prepared as described above, washed once, and diluted to an appropriate concentration with Eisen's medium. The number of plaque-forming cells was determined according to the method of Cunningham and Szenberg (16). A rabbit anti-mouse gamma-globulin serum was added to the reaction mixture in order to assay for developed plaques. The number of indirect plaques was derived by difference from the numbers of plaques obtained in the presence or absence of antiglobulin antibody.

Graft-versus-host assays. An estimate of the graft-versus-host activity of thoracic duct lymphocytes was obtained by the method of Simonsen (17). The cells were injected into the peritoneal cavity of neonatal F_1 hybrid mice within 18 hr of birth. The recipients were killed 10 days later in order to determine the splenic indices, that is, the ratio of spleen to body weight of each recipient injected with one group of parental cells divided by the average spleen to body weight ratio of all the recipients injected with syngeneic F_1 cells.

Statistical analyses. The geometric means and standard errors of the mean were

calculated from the log $_{10}$ of the plaque-forming cell counts. In the graft-versus-host assays the arithmetic mean and the standard errors of the mean were calculated from the splenic indices. p values were determined by the Rank test and, in the comparison of the means of any two groups of observations, a significance level of 0.05 was chosen.

Experimental procedure. To investigate the possible recruitment of lymphocytes in mice stimulated with sheep erythrocytes, the following experimental design was used. Thoracic duct fistulas were established in 10–25 mice in the afternoon and the lymph allowed to drain overnight. Lymphocytes were collected and pooled the next morning and 10 million cells were injected intravenously together with 4×10^8 sheep erythrocytes and 4×10^8 horse erythrocytes into thymectomixed, irradiated, marrow-protected mice. The donors of the thoracic duct cells were then divided equally into two groups: mice in one group received an intravenous injection of 4×10^8 sheep erythrocytes while mice in the other group received 0.1 ml saline intravenously. Overnight collections of lymphocytes were made 1, 2, 3, and 5 days after these injections. Cells from mice of each group were pooled separately and 10 million cells were injected intravenously together with 4×10^8 sheep erythrocytes and 4×10^8 horse erythrocytes into thymectomized, irradiated, and marrow-protected mice. Previous results had established that the peak direct plaque-forming cell response of thymectomized, irradiated recipients of thoracic duct cells and sheep erythrocytes occurred 6 days after injection (18). Based on these findings and on other results (unpublished), it was decided to determine the number of direct and indirect plaque-forming cells per spleen to both sheep and horse erythrocytes 6 days after cell transfer and challenge. The capacity of thymectomized irradiated mice to respond to sheep erythrocytes, when not given lymphocytes, is well below that of nonthymectomized, irradiated controls (19). Nevertheless, such thymectomized mice do give a measurable response at 6 days when challenge is made 4 weeks postirradiation (Table 1). This "background" number of plaque-forming cells was subtracted from the number of plaque-forming cells obtained in spleens of thymectomized, irradiated recipients of thoracic duct cells and sheep and horse erythrocytes.

An essentially similar approach was used to investigate the possible recruitment of lymphocytes in animals given histoincompatible cells. An intravenous injection of 50 million spleen cells from 7-week-old (CBA × C57BL)F_1 mice was given to

TABLE 1

PLAQUE-FORMING CELL RESPONSE OF THYMECTOMIZED, IRRADIATED, MARROW-PROTECTED CBA MICE INJECTED WITH SHEEP AND HORSE ERYTHROCYTES ONLY

Assay	Number of mice in group	Number of plaque-forming cells per spleen at 6 days [a]	
		Anti-sheep erythrocytes	Anti-horse erythrocytes
Direct plaques	9	2770 (3770–2030)	610 (910–400)
Indirect plaques	9	880 (1240–520)	230 (360–150)

[a] Results are expressed as geometric means and the upper and lower limits of the SE are shown in brackets.

10–15 normal CBA mice. Control CBA mice were given saline. Thoracic duct fistulas were then established within 1 hr in mice of each group and lymph was collected over 12-hr periods for up to 5 days. One or 5 million thoracic duct cells from successive collections from mice of each group were injected intraperitoneally into newborn (CBA × C57BL)F_1 or (CBA × BALB/c)F_1 mice. These were killed 10 days later and their spleen weights measured to assay for graft-versus-host activity. A splenic index of 1.5 or over was taken as evidence of graft-versus-host activity.

The thoracic duct lymphocyte output of mice stimulated with antigen was not found to be significantly different from that of unstimulated mice in the experiments reported here.

RESULTS

The results, which are illustrated in Figs. 1–4 and statistically analyzed in Tables 2 and 3, can be summarized as follows.

Activity of thoracic duct lymphocytes from mice given sheep erythrocytes. Thoracic duct lymphocytes, collected from saline-treated mice at various intervals over a period of 5 days, were equally effective in adoptively transferring to thymectomized, irradiated recipients the capacity to respond to either sheep or horse erythrocytes. Thus, approximately similar numbers of direct (Fig. 1) and indirect (Fig. 2) plaque-forming cells to either sheep or horse erythrocytes were obtained in spleens of mice injected with lymphocytes from different pools. In general, the response to horse erythrocytes tended to be somewhat lower than that to sheep.

Thoracic duct lymphocytes obtained from mice injected with sheep erythrocytes 1–2 days before did not enable thymectomized, irradiated recipients to produce a direct (Fig. 1) or indirect (Fig. 2) plaque-forming cell response to sheep erythrocytes significantly above that found in control thymectomized mice not given lymphocytes. The deficiency in the particular lymphocyte population used was specific since the same population allowed thymectomized mice to respond to horse erythrocytes as effectively as mice given lymphocytes from saline-treated donors. Capacity to transfer responsiveness to sheep erythrocytes had returned by 3 days and the direct plaque-forming cell response in recipients of the 5-day thoracic duct cell collection was of the same order of magnitude whether the donors were treated with sheep erythrocytes or saline. On the other hand, a greatly increased number of indirect plaque-forming cells to sheep erythrocytes was obtained in the spleens of mice receiving the 5-day lymphocyte collection from sheep erythrocyte-treated donors.

Both the direct and indirect plaque-forming cell response to horse erythrocytes in thymectomized, irradiated recipients of thoracic duct cells from sheep erythrocyte-treated donors were comparable to the responses obtained in recipients of lymphocytes from saline-treated mice when the cells were obtained 1–2 days postcannulation but considerably below when obtained at 5 days (Figs. 1 and 2).

The above data were pooled from three experiments. A further six experiments were performed and gave similar results.

Activity of thoracic duct lymphocytes from mice given histoincompatible cells. Preliminary experiments showed that 1 million thoracic duct lymphocytes from

TABLE 2

PLAQUE-FORMING CELL RESPONSE OF THYMECTOMIZED, IRRADIATED, MARROW-PROTECTED CBA MICE GIVEN SHEEP AND HORSE ERYTHROCYTES AND THORACIC DUCT LYMPHOCYTES TAKEN FROM CBA DONORS AT VARIOUS INTERVALS OF TIME AFTER INJECTION OF SHEEP ERYTHROCYTES OR SALINE [a]

Lymphocyte collection	Number of mice in group	Erythrocytes used in assay	Number of plaque-forming cells per spleen at 6 days [b]				
			Direct plaques	p values	Indirect plaques	p values
Uninjected donors	9	Sheep	64270 (70980–58200)	<.01	38010 (53360–27070)	<.01
1 Day after injection of sheep erythrocytes	7	Sheep	10070 (15040–6740) [c]		2140 (4110–1100) [c]	
1 Day after injection of saline	6	Sheep	78790 (91690–67710)	<.01	150350 (172390–131130)	<.01
2 Days after injection of sheep erythrocytes	7	Sheep	4500 (6250–3250) [c]	<.005	12870 (23360–7090)	<.01
2 Days after injection of saline	6	Sheep	54490 (63000–47120)		144400 (163200–127760)	
3 Days after injection of sheep erythrocytes	6	Sheep	33980 (45500–25380)	<.05	38990 (52730–28830)	NS [d]
3 Days after injection of saline	7	Sheep	93790 (117340–74970)		96140 (133220–63990)	
5 Days after injection of sheep erythrocytes	6	Sheep	78450 (86890–70820)	NS	1652840 (1952490–1399180)	<.01
5 Days after injection of saline	5	Sheep	97220 (104930–90070)		200970 (249560–161840)	
5 Days after injection of sheep erythrocytes	6	Horse	19420 (25430–14830)	<.01	19420 (25430–14830)	<.01
5 Days after injection of saline	5	Horse	61020 (66730–55810)		109190 (128580–92720)	

[a] Detailed results are shown in Figs. 1 and 2. In this Table, the number of plaque-forming cells obtained in thymectomized, irradiated mice not given lymphocytes is not subtracted from the values shown.

[b] Results are expressed as geometric means and the upper and lower limits of the SE are shown in brackets.

[c] These values are not significantly different from those obtained in control thymectomized mice not given lymphocytes (cf. Table 1).

[d] NS = not significant.

TABLE 3

SPLENIC INDICES OF 10-DAY-OLD (CBA × C57BL)F_1 MICE INJECTED AT BIRTH WITH CBA THORACIC DUCT LYMPHOCYTES TAKEN AT VARIOUS INTERVALS OF TIME AFTER INJECTION OF (CBA × C57BL)F_1 SPLEEN CELLS OR SALINE

Lymphocyte collection	Number of mice in group	Splenic indices (± SE)	p values
Uninjected donors	10	2.71 ± 0.22	<.01
36 Hr after injection of F_1 spleen cells	7	1.70 ± 0.11	<.05
36 Hr after injection of saline	8	2.34 ± 0.17	
48 Hr after injection of F_1 spleen cells	10	1.29 ± 0.09	<.01
48 Hr after injection of saline	8	2.26 ± 0.15	
120 Hr after injection of F_1 spleen cells	3	4.86 ± 0.07	<.05
120 Hr after injection of saline	4	3.23 ± 0.12	

CBA mice injected intraperitoneally into (CBA × C57BL)F_1 or (CBA × BALB/c)F_1 hybrid baby mice produced splenic indices of the order of 1.7, but considerable variation was found. On the other hand 5 million thoracic duct cells produced significantly high splenic indices (around 2.8) with little variation. The dose of 5 million cells was thus chosen for all experiments.

Thoracic duct cells, obtained at various intervals of time from saline-treated donors, did not produce any significant variation in splenic indices (Figs. 3 and 4). By contrast, lymphocytes taken from CBA mice 2 days after injection of (CBA × C57BL)F_1 cells gave no evidence of graft-versus-host activity in (CBA × C57BL)F_1 mice as determined by the magnitude of the splenic index but, when obtained 5 days after injection, caused a marked increase in the splenic index to a

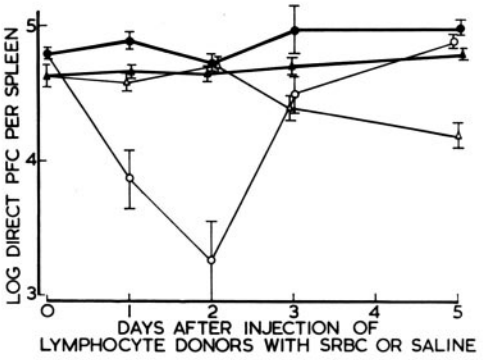

FIG. 1. Direct plaque-forming cell response of thymectomized, irradiated, marrow-protected CBA mice given sheep and horse erythrocytes and thoracic duct lymphoctes. ○—○ = anti-sheep erythroyte plaques in recipients of lymphoctyes from donor mice given sheep erythrocytes on day 0; △—△ = anti-horse erythrocyte plaques in same recipients; ●—● = anti-sheep erythrocyte plaques in recipients of lymphocytes from donor mice given saline on day 0; ▲—▲ = anti-horse erythrocyte plaques in same recipients. Geometric means of results obtained from five to nine mice per point are shown and the vertical bars indicate the upper and lower limits of the SE. The respective means of the plaques obtained in control thymectomized, irradiated mice not given lymphocytes was subtracted in every case.

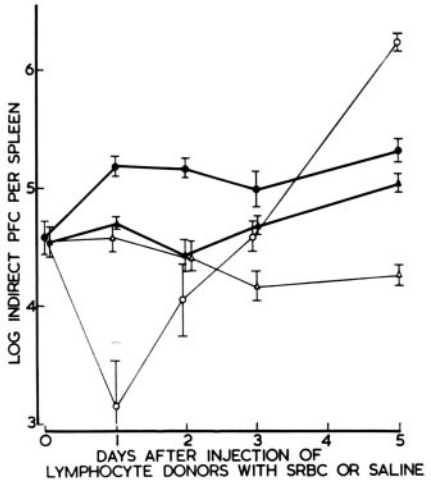

FIG. 2 Indirect plaque-forming cell response of thymectomized, irradiated, marrow-protected CBA mice given sheep and horse erythrocytes and thoracic duct lymphocytes. Symbols are used as for Fig. 1.

level significantly above that given by cells from saline-treated donors (Fig. 3). The specificity of this effect is demonstrated by an absence of variation in splenic indices obtained in neonatal (CBA × BALB/c)F_1 recipients of lymphocytes taken at various intervals of time after injection of the donors with (CBA × C57BL)F_1 cells (Fig. 4).

DISCUSSION

The results of the present experiments show that the capacity of a circulating lymphocyte population to transfer responsiveness towards a particular antigen is markedly and specifically depressed 1–2 days after injection of antigen, is restored towards normal levels by day 3, and is considerably enhanced by day 5. These findings can be interpreted in terms of a recruitment of antigen-sensitive cells from a pool of recirculating lymphocytes, occurring soon after antigen administration, and followed in turn by a rapid reentry of such cells into the pool in an increased proportion. The observations were made with two different antigenic systems in mice: heterologous erythrocytes and histoincompatible spleen cells. It is possible, therefore, that recruitment of specifically reactive cells may be a universal sequel of antigen challenge.

What explanation can be offered to account for the period of unresponsiveness? Ford (20) studied the effects of transferring rat thoracic duct lymphocytes to irradiated rats at varying intervals of time after injecting the recipients with sheep erythrocytes. He observed that a delay in the transfer of lymphocytes of more than 12 hr significantly decreased the peak hemolysin response and increased the latent period taken to reach the peak. He suggested that the inductive influence of antigen waned steadily, presumably as a result of antigen processing by macrophages, and predicted that "the effective period of recruitment probably lasts 1–2 days." This suggestion obtains support from the experiments reported here and implies

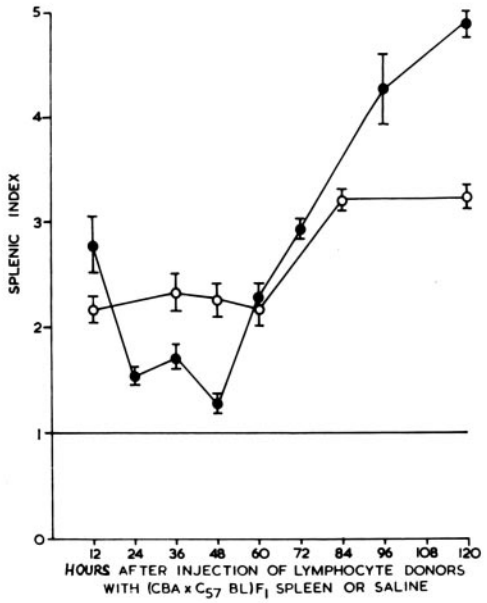

FIG. 3. Splenic indices of 10-day-old (CBA × C57BL)F_1 mice injected at birth with (a) thoracic duct lymphocytes from CBA mice taken at various intervals of time after injection of (CBA × C57BL)F_1 spleen cells (●—●), or (b) thoracic duct lymphocytes from CBA donors given only saline (○—○). Each point represents the arithmetic mean of determinations made on 3–10 mice (usually 6), and the vertical bars represent the magnitude of twice the SEM. The difference in the means of the control values (o) are not statistically significant. Statistical evaluation of differences between control and experimental values is given in Table 3.

that, if adequate amounts of antigen persisted in an immunogenic form, the period of recruitment might be prolonged.

It seems that termination of the period of unresponsiveness may be associated with the emergence of blast cells from the antigen-stimulated lymphoid organs. Several studies have demonstrated the presence of such cells in the lymphatics or thoracic duct of animals 3–5 days after antigenic stimulation (21, 22), and it was suggested that these cells were responsible for propagating the immune response from regionally stimulated lymph nodes to the rest of the lymphoid system (23). The period of unresponsiveness following administration of antigen can therefore be considered as the period during which specific antigen-sensitive cells are withdrawn from the circulation into antigen-stimulated lymphoid tissues where several events take place among which can be listed: interaction with antigen, blast transformation of small lymphocytes, cell division and generation of more lymphocytes (24), and collaboration between T and B cells (25). Termination of the period of unresponsiveness in the circulating lymphocyte population is probably associated with the release of the progeny of the antigen-activated lymphocytes from the regionally stimulated lymphoid tissues.

In the experiments reported here, it is of interest to note that although unresponsiveness of the circulating lymphocyte pool was clearly specific during the pe-

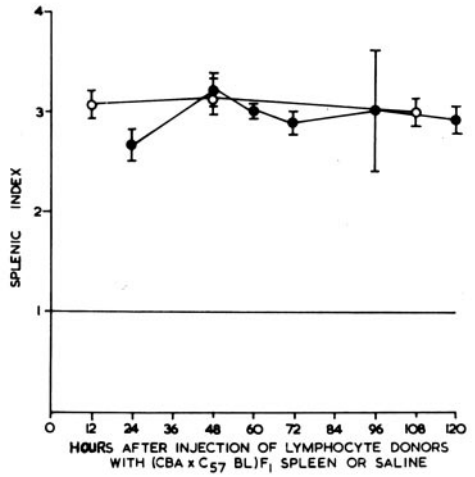

FIG. 4. Splenic indices of 10-day-old (CBA × BALB/c)F_1 mice injected at birth with (a) thoracic duct lymphocytes from CBA mice taken at various intervals of time after injection of (CBA × C57BL)F_1 spleen cells (●—●); or (b) thoracic duct lymphocytes from CBA donors given only saline (○—○). Each point represents the arithmetic mean of determinations made on 3–10 mice (usually 6) and there is no statistically significant difference between any of the values shown here.

riod of recruitment, the capacity of lymphocytes taken 3–5 days after injection of sheep erythrocytes to transfer immune responsiveness to horse erythrocytes was decreased. A similar decrease was not noted when histoincompatible spleen cells were used as the antigen. Antigenic competition could be invoked as an explanation of the findings obtained with heterologous erthrocytes. Such a competition occurs only if the foreign erythrocytes are given at different times: thus a secondary response to one antigen markedly depresses the primary response to another (26, 27).

A recruitment effect similar to the one described here has been observed in the rabbit (28). In this species, appreciable numbers of immunocompetent cells, capable of transferring responsiveness to heterologous erythrocytes, are present in the marrow. It was found that 8 hr after antigen administration, the marrow became specifically unresponsive for a transient period of time lasting about 40 hr. The investigators accounted for their findings by postulating a migration of specific immunocompetent cells from the marrow to other sites. It would be interesting, in the light of our results, to determine whether the immunocompetent lymphocytes found in rabbit bone marrow form part of a recirculating lymphocyte pool and whether they are capable of being removed by prolonged thoracic duct drainage. The contamination of bone marrow in rats by cells of the recirculating lymphocyte pool has been clearly demonstrated (29).

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