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Distribution of plaque-forming cells in the mouse for a protein antigen

EVIDENCE FOR HIGHLY ACTIVE PARATHYMIC LYMPH NODES FOLLOWING INTRAPERITIONEAL INJECTION OF HEN LYSOZYME

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Summary. The distribution of plaque-forming cells (PFC) throughout the lymphoid system of CBA mice was followed with time after a primary intraperitoneal injection of hen egg white lysozyme emulsified in Freund's complete adjuvant (HEL-CFA) and after a secondary soluble injection. Throughout the primary response (predominantly IgG) and during the first week of the secondary response (exclusively IgG), the highest density of PFC was found in the draining parathymic lymph nodes, followed by the local spleen and mesenteric lymph nodes. The antibody-forming activity of the bone marrow increased as the immune response progressed, so that by the 3rd week of the secondary response this compartment provided the majority of the PFC. PFC first appeared in the accessory axillary, brachial or inguinal lymph nodes and in the thymus a few days after the secondary injection but accounted for only 1–5 per cent of the total activity during the entire course of the secondary response.

The specificity of the antibody produced in the spleen, parathymic and mesenteric lymph nodes was identical as judged by plaque inhibition by seven chemically related lysozymes which implies that these PFC were well mixed. It is postulated, therefore, that the change in distribution of PFC from an early local response to a general systemic response, and finally to a predominantly bone marrow response, was due to the migration of memory cells from the draining parathymic lymph nodes and spleen throughout the lymphoid system with an ultimate settling of the cells in the bone marrow.

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INTRODUCTION

The magnitude of an immune response following antigenic challenge is usually measured by determining the amount of specific antibody which is produced. In the most commonly studied locations of the spleen and serum, differences are evident in the rates of appearance and decay of antibody during the course of this immune response. On any one day there may not be a positive quantitative correlation between the amount of serum antibody and the number of splenic PFC, especially at later stages of the response when PFC are dropping rapidly while serum titres remain elevated (Anderson and Dresser, 1972; Chaperone, Selner and Claman, 1968). This lack of correlation is difficult to reconcile with the half-lives in the mouse of the passively transferred IgM, IgF(γ1), IgG(γ2a), IgG(γ2b) and IgA of 0·5, 5·1, 2·7, 4·0 and 1·2 days, respectively (Fahey and Sell, 1965). These observations suggest that PFC which contribute to the circulating pool of antibody are being sequestered in other parts of the lymphoid system.

Indeed, the general observation of most investigators is that immediately after antigenic challenge the draining lymph nodes or local lymphoid tissues are most active in antibody formation, while the memory cells migrate to accessory lymphoid compartments as the immune response develops as demonstrated *in vitro* in the guinea-pig and rabbit (Askonas and Humphrey, 1958; Askonas and White, 1956; Fleming, Wilkinson and White, 1967).

The *in vivo* distribution of PFC has been studied primarily with the use of sheep erythrocytes as the challenge antigen. The findings are that, in the mouse, the spleen is the major source of antibody production early in the response for both IgM and IgG. As the response progresses, the bone marrow surpasses the spleen and becomes a major source of PFC (Anderson and Dresser, 1972; Chaperone *et al.*, 1968; Mellbye, 1971; Benner, Meima, van der Meulen and van Muiswinkel, 1974; Benner, Meima and van der Meulen, 1974; Eidinger and Pross, 1967). These extra-splenic sites which become more important as the immune response progresses, make it apparent that on any one day the spleen may not characterize accurately the overall immune status of the mouse.

It was important to understand the kinetics of PFC production and to locate the most active antibody-forming sites in response to a protein antigen, because there are two reasons why these parameters might differ for protein antigens as compared to particulate antigens. First, it is not surprising that much of the anti-erythrocyte immunological activity takes place in the spleen where there is a tendency for particulate antigens to become trapped (Wagner, Razzak, Gaertner, Caine and Feagin, 1962). Secondly, IgM is the predominant class of antibody produced in response to a primary injection of erythrocytes and there is a substantial boost in this class after a secondary injection (Sercarz and Byers, 1967). On the other hand, in response to a protein antigen, the IgG classes account for the majority of the antibody produced. The relationship between the ratio of IgM to IgG produced may reflect differences in the PFC populations or accessory cell populations involved, which may vary in different lymphoid compartments.

The distribution of PFC was followed in the spleen, thymus, bone marrow and liver, and in the parathymic, inguinal, axillary, brachial and mesenteric lymph nodes of CBA mice during the primary and secondary responses. The parathymic lymph nodes were chosen because there was evidence that they might be immunologically active after an intraperitoneal injection (Leckband and Boyse, 1971; Mauss and Schmitt-Slomska, 1974). The other locations were chosen because they are generally believed to offer a major immunological contribution.

Both the density of PFC and contribution to the total antibody-forming activity were evaluated. These cellular responses were then correlated with serum antibody titres for the same time periods. A comparison of the specificity of PFC found in different tissues was made by looking at the patterns of inhibition of plaques by seven chemically related lysozymes in small pieces of the spleen, mesenteric and parathymic lymph nodes.

MATERIALS AND METHODS

Mice

The inbred strain CBA/J was purchased from the Jackson Laboratory, Bar Harbor, Maine. The mice were maintained on Purina lab chow and water *ad libitum*. Male and female mice were used at the age of 3–4 months.

Immunizations

The immunization regime included a primary intraperitoneal injection of 50 μg of hen egg white lysozyme (Societa Produtti Antibiotica, Milano) emulsified in complete Freund's adjuvant, containing *M. butyricum* (Baltimore Biological Labs, Cockeysville, Maryland). For the secondary injection, the mice received 100 μg soluble protein intraperitoneally 35 days after the initial injection.

Cell suspensions

At various times following the primary and secondary injections groups of five mice were killed for cellular studies by exsanguination. The spleens, lymph nodes (axillary, brachial, inguinal, mesenteric and parathymic), thymuses and livers were removed immediately and cell suspensions prepared by gently teasing each organ sequentially through coarse 80-mesh and fine 250-mesh stainless steel screens into cold HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulphonic acid) buffered Eagle's minimum essential medium (Grand Island Biological Company) containing 0·25 per cent gelatin (Atlantic Gelatin, Woburn, Maine). Bone marrow was obtained by first flushing the shafts of the femoral and tibial

bones with cold medium and then passing the cells through a fine 250-mesh screen. All cell suspensions were washed twice and resuspended in cold medium.

Haemolytic plaque assay

The basic procedure of Cunningham and Szenberg (1968) was used. Hen lysozyme was coupled to goat erythrocytes (Colorado Serum, Denver, Colorado) with 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide HCl and the coupled red cells were suspended to 10 per cent and used at a final concentration of 1·25 per cent. IgM-forming cells were calculated by direct plaque assay in which fresh guinea-pig serum, absorbed with goat erythrocytes at 0°, was used as a source of complement at a final dilution of 1:16. IgG-forming cells were calculated by indirect plaque assay in which, in addition to guinea-pig serum, squirrel monkey anti-mouse immunoglobulin was added at a final dilution of 1:400 (facilitated plaques). The facilitating serum was inactivated at 56° and absorbed with goat erythrocytes before use.

Plaque inhibition assay

In order to study the specificity of the antibody produced by the plaque-forming cells, the inhibitory capacity of hen (HEL), bob-white (BEL), duck (DEL), Japanese quail (JEL), peafowl (PEL), ringed-neck pheasant (REL) and turkey (TEL) egg white lysozymes was studied. With the exception of hen lysozyme, these lysozymes were isolated from egg white by column chromatography on carboxymethylated Sephadex (Pharmacia) followed by further purification on the weak cation exchanger, Bio-Rex-70 (Bio-Rad, Richmond, California). The amount of inhibition was measured by adding soluble lysozyme to the above reaction mixture. Results are presented as percentage decrease found on slides containing inhibitor compared to slides with no inhibitor. Fifty micrograms per millilitre usually represented a plateau level of inhibitor.

Antigen binding capacity

Hen lysozyme was iodinated by a chloramine-T procedure (McConahey and Dixon, 1966). Five microlitres of a series of dilutions of the test antisera (diluted 1/5, 1/25 and 1/125 in normal mouse serum) were added to 20 μl of borate buffer, pH 8·4, containing cytochrome-c at 5 mg/ml (to control nonspecific binding). To this was added 10 μl of iodinated HEL at 0·01 μg/ml. After 2 h in the cold, the antigen–antibody complexes were precipitated with a rabbit anti-mouse myeloma 5563 protein. After a 1 h incubation at room temperature the precipitates were centrifuged and washed once in borate buffer. Antigen-binding capacity was determined at 33 per cent binding of antigen, and then converted to be expressed as μg of HEL bound per millilitre of serum.

Isoelectric focusing

The sera were tested by isoelectric focusing (IEF) using the basic procedure of Awdeh, Williamson and Askonas (1968), as modified by Cecka and Miller in our laboratory (unpublished). The sera were focused in 5 per cent polyacrylamide containing pH 5–8 carrier ampholytes (LKB Produkter AB, Sweden) in an electric field of 200 V over a distance of 8 cm for 20 h. After the antibody molecules were separated according to their isoelectric points, they were exposed to iodinated lysozyme. After the gels had been dried onto the supporting plates, the bands of antibody that bound the iodinated lysozyme were visualized by autoradiography using Kodak No Screen X-Ray film.

RESULTS

Primary cellular response

The primary PFC response was studied by following the distribution of active cells in the spleen, thymus, bone marrow, mesenteric lymph nodes, parathymic lymph nodes and a pool of the inguinal, brachial and axillary lymph nodes for 7, 14, 21 and 30 days after an intraperitoneal injection of 50 μg HEL-CFA. Table 1(a) and (b) summarizes the concentration and the total number of PFC in the various tissues with time. In order to calculate the total bone marrow response, use was made of the estimates of Chervenick, Boggs, Marsh, Cartwright and Wintrobe (1968) who determined that one femur of the mouse represents about 5·9 per cent of the total number of bone marrow cells.

After a very strong initial response, the number of PFC drops steadily. The concentration of PFC is greatest in the draining parathymic lymph nodes, followed by the local mesenteric lymph nodes and then spleen and bone marrow. The organ making the greatest contribution to the total response fluctuates from the mesenteric lymph nodes early in the response to a broader contribution by spleen,

Table 1. Primary plaque-forming cell (PFC) response

(a) Concentration of PFC,*†

Day after injection	Bone marrow	Lymph nodes‡	Parathymic lymph nodes	Mesenteric lymph nodes	Spleen	Thymus	Serum titres§ (μg/ml)
7	4 (0)¶	1 (0)	3300 (27)	2400 (17)	170 (14)	1 (0)	1
14	10 (0)	1 (0)	203 (0)	124 (0)	26 (2)	0 (0)	18
21	5 (0)	0 (0)	87 (0)	28 (1)	7 (11)	0 (0)	75
30	2 (0)	0 (0)	58 (0)	16 (0)	1 (3)	0 (0)	87

(b) Total number of PFC

Day after injection	Bone marrow‖	Lymph nodes‡	Parathymic lymph nodes	Mesenteric lymph nodes	Spleen	Thymus
7	250 (0)	1 (0)	11,300 (90)	44,000 (320)	9000 (700)	20 (0)
14	620 (0)	1 (0)	1000 (0)	2400 (0)	2680 (180)	0 (0)
21	390 (0)	0 (4)	235 (0)	340 (10)	780 (1300)	0 (0)
30	180 (0)	0 (0)	160 (0)	270 (0)	100 (420)	0 (0)

* PFC/10^6.
† Responses of pool of five mice per time period.
‡ Pool of axillary, brachial and inguinal lymph nodes.
§ μg HEL bound per millilitre of serum. Calculated from ABC–33 per cent at 0·01 μg HEL (reaction volume 35 μl).
¶ First number is indirect PFC response and number in parentheses is the direct PFC response.
‖ Bone marrow calculation based on estimate that one femur represents 5·9 per cent of bone marrow cells (Chervenick *et al.*, 1968).

mesenteric lymph node, parathymic lymph node and bone marrow later in the response. An insignificant number of PFC were detected in the thymus and in the accessory axillary, brachial and inguinal lymph nodes during this 30-day primary period, suggested that migration of mature PFC to these peripheral sites has not occurred.

Each cell population was tested for its ability to produce indirect and direct plaques with the use of a squirrel monkey anti-mouse immunoglobulin antiserum. By 7 days after the primary injection, a few direct PFC were detected in the spleen, mesenteric and parathymic lymph nodes. After this time, the spleen provided the majority of the total direct plaques. As opposed to the anti-erythrocyte primary response, during which the IgM antibody class dominates, less than 4 per cent of all of the plaques were direct in the anti-HEL primary response.

Primary humoral response

The primary humoral response follows an almost inverse relationship to the total PFC response. The serum HEL-binding titre at day 7 is very weak (1 μg/ml) and increases steadily to 87 μg/ml by day 30 (Table 1a). By studying the isoelectric focusing patterns of the sequential sera from a second group of individual CBA mice not killed for cellular studies (Fig. 1), it can be seen that a few new clonotypes appear as the response progresses but, in general, the clonotypes already present expand, yielding more antibody and a stronger band intensity. This suggests that there is an accumulation of antibody from the clones of PFC already present, or that the rapidly produced clones of the PFC detected in the organs studied may be migrating from the fixed lymphoid tissue into the peripheral organs. This type of migration has been reported to occur in mice and rabbits where the peripheral blood contributed 1/16 to 1/30 the concentration of PFC found in the spleen after a primary injection of sheep erythrocytes (Landy, Sanderson, Bernstein and Jackson, 1964; Sorkin and Landy, 1965; Rabin and Rose, 1973).

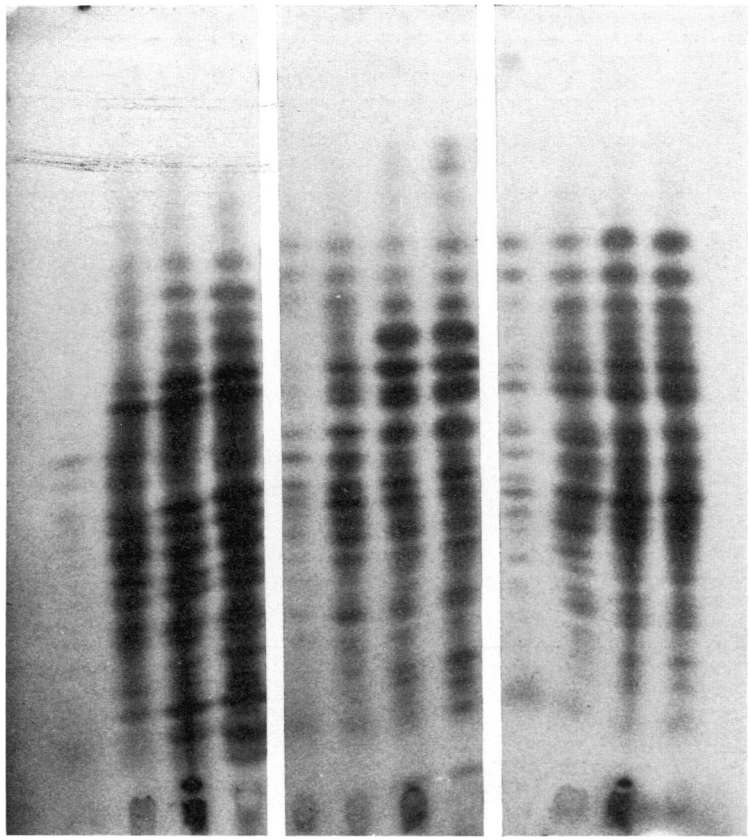

Figure 1. Maturation of the primary serum response. IEF patterns of anti-HEL antibody obtained 7, 14, 21 and 30 days after primary injection of HEL-CFA in three representative CBA/J mice.

Secondary cellular response

Kinetics

The secondary immune response was studied by following the distribution of PFC at the same sites for several time points following a secondary intraperitoneal injection of 100 μg soluble HEL given 35 days after the primary injection.

In Table 2(a) and (b) are summarized the concentration and the total number of PFC in the various tissues with time after the secondary injection. As opposed to the primary response, no direct plaques were ever observed, even in the spleen, so that all the numbers in this Table represent indirect (facilitated) PFC.

There is a wide distribution of PFC activity throughout the lymphoid system after the secondary injection of HEL. A response is already evident 1 day after the secondary boost, but this may be due to residual PFC which were generated during the primary response. By 3 days, the cellular response is strong and continues to increase to a peak on the fourth to fifth days.

Lymph node contribution

From 2 to 14 days, the parathymic lymph nodes contained the highest concentration of PFC followed in general by the spleen, mesenteric lymph nodes and bone marrow. For the first time, the accessory axillary, brachial and inguinal lymph nodes started to contribute PFC after a slight lag of 1 or 2 days with respect to the other lymphoid organs. Because these accessory lymph nodes contained no PFC activity during the primary response, this suggests that memory cells had migrated to these sites and were not expressed until a secondary exposure to

Table 2. Secondary plaque-forming cell (PFC) response

(a) Concentration of PFC,*†

Day after injection	Bone marrow	PT-LN‡	Thymus	Spleen	Axill. LN	Brach. LN	Inguin. LN	Mesent. LN	Liver	Serum titres§ (μg/ml)
1	5	0	0	3	0	0	0	5	n.t.¶	3
2	7	64	1	3	0	0	0	2	n.t.	5
3	14	647	0	190	12	10	13	114	3	9
4	243	3130	2	990	80	68	84	1030	0	53
5	279	4230	18	550	44	29	19	3480	1	127
6	470	2800	25	470	270	180	76	2040	0	380
7	460	1970	16	180	31	53	5	465	0	570
8	224	1920	4	120	44	40	26	525	n.t.	470
9	292	1680	11	89	40	13	16	400	0	490
12	299	500	9	50	10	2	4	160	n.t.	390
14	250	320	4	20	8	1	1	87	n.t.	210
21	185	10	6	2	3	1	1	11	n.t.	61

(b) Total number of PFC

Day after injection	Bone marrow	PT-LN‡	Thymus	Spleen	Axill. LN	Brach. LN	Inguin. LN	Mesent. LN	Liver
1	220	0	0	460	0	0	0	150	n.t.¶
2	240	166	12	620	0	0	0	50	n.t.
3	1160	1290	0	24,740	90	80	60	3100	72
4	45,440	6890	80	237,000	1200	680	810	113,200	0
5	38,460	22,860	490	112,000	290	350	130	111,400	24
6	59,220	6990	1780	126,700	3750	3630	830	65,200	0
7	52,440	5910	500	37,800	260	840	40	17,900	0
8	21,200	5760	400	34,800	840	820	210	16,700	n.t.
9	19,230	5040	640	20,000	410	270	130	9200	0
12	18,240	1518	450	14,500	100	45	30	6500	n.t.
14	15,500	960	230	3660	80	20	7	3460	n.t.
21	8340	350	440	146	30	22	8	143	n.t.

* PFC/10^6.
† Responses of pool of five mice per time point.
‡ PT-LN = parathymic lymph nodes.
§ HEL (μg bound per millilitre of serum).
¶ n.t. = Not tested.

soluble antigen. These three pairs of lymph nodes represent about 1/4 to 1/3 of the total lymph node mass in the mouse (E. Cooper, personal communication), and assuming that other lymph nodes provide, in sum, an equivalent number of PFC, the total contribution of all the accessory lymph node organs would only account for 4 per cent of the total response.

Bone marrow, thymus and liver contribution

Very late in the response, the bone marrow surpassed all the tissues in the concentration of PFC. This was due, in part, to the drop with time in the concentration of PFC in the other organs as compared to the persistent production of PFC by the bone marrow.

There were a few PFC detected in the thymus during the secondary response which in terms of total contribution, generally equalled that of one set of accessory lymph nodes. It may be possible that there was contamination of the thymus cells by the neighbouring highly active parathymic lymph nodes. However, PFC have been reported to be present in the thymus (Anderson and Dresser, 1972; Chaperone *et al.*, 1968; Benner, Meima, van

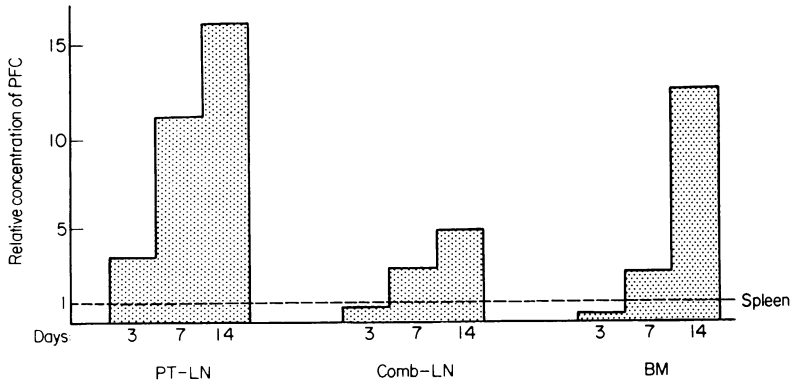

Figure 2. Development of the relative density of PFC in three major immunological compartments at 3, 7 and 14 days after the secondary injection of soluble HEL. All values are normalized to the concentration of splenic PFC. PT-LN = parathymic lymph nodes; Comb-LN = combined axillary, brachial, inguinal and mesenteric lymph nodes; BM = bone marrow.

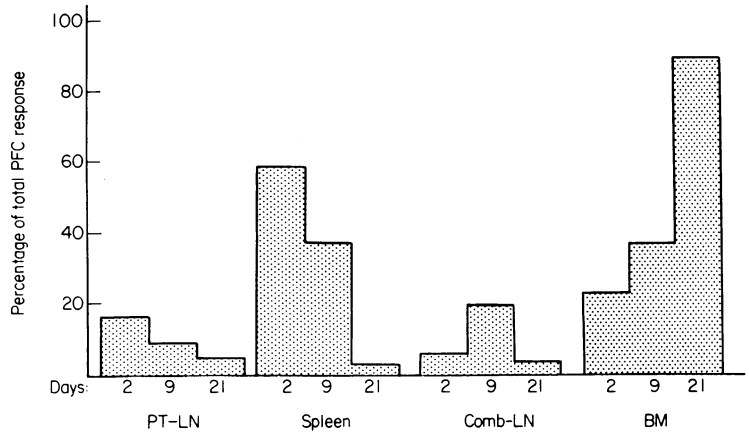

Figure 3. Development of the percentage of total PFC contributed by four major immunological compartments at 2, 9 and 21 days after the secondary injection of soluble HEL. Abbreviations are the same as in Fig. 2.

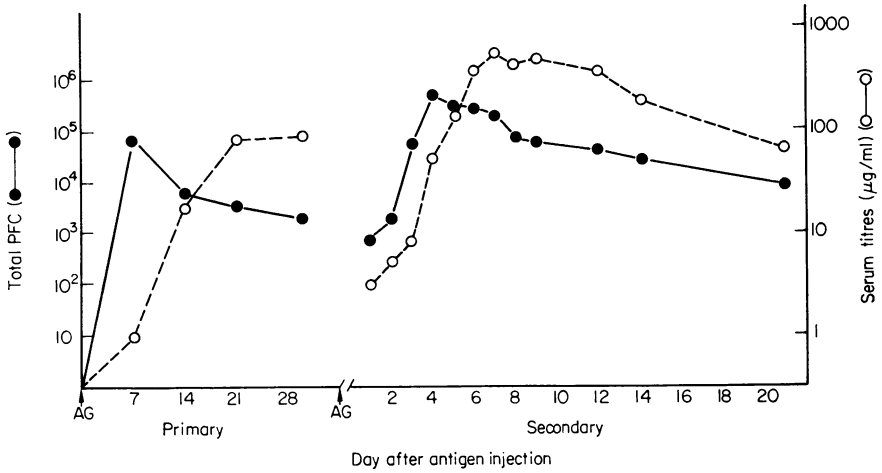

Figure 4. Kinetics of the cellular and humoral anti-HEL antibody response. Secondary injection of soluble HEL was given 35 days after the primary injection of HEL-CFA. Total PFC include the contributions from all compartments tested. Serum titres are expressed as micrograms of HEL bound per millilitre of serum.

Table 3. Inhibition of plaque-forming cells in various tissues

(a) Inhibition of plaque-forming cells (PFC) obtained from whole organs 3 days after secondary injection

Tissue studied	PFC/slide no inhibitor	Percentage inhibition by:*						
		HEL	BEL	DEL	JEL	PEL	REL	TEL
Spleen	245	100	97	37	94	90	46	78
PT-LN	177	100	99	40	95	88	58	88
Mesent.-LN	320	99	97	39	97	91	60	77

(b) Inhibition of plaque-forming cells (PFC) obtained from small segments of organs 3 days after secondary injection

Segment studied	PFC/slide no inhibitor	Percentage inhibition by:*						
		HEL	BEL	DEL	JEL	PEL	REL	TEL
Spleen-1†	367	99	99	45	95	91	60	84
Spleen-2	197	100	98	43	93	87	48	82
PT-LN-1‡	80	100	95	40	92	88	52	84
PT-LN-2	114	100	96	35	90	84	59	86
Mesent.-LN-1§	100	100	96	38	92	91	58	90
Mesent.-LN-2	62	100	94	45	89	87	54	86

* Final concentration of inhibitor was 50 μg/ml.
† Small pieces obtained from opposite ends of the spleen.
‡ One small node and half of lower larger node.
§ Small pieces obtained from opposite ends of the mesenteric lymph node.

der Meulen and van Ewijk, 1974), and the data of these investigators indicate that along with bone marrow, the thymus becomes an important PFC generating compartment very late in the response.

A negligible number of PFC were found in the liver which may have been due to technical problems because much difficulty was encountered in trying to prepare a single cell liver suspension. Garvey and Linker-Israeli (1972) have found a considerable number of anti-erythrocyte plaque-forming hepatocytes in mice and rabbits after a primary injection.

Proportion of responsiveness in different lymphoid compartments

Fig. 2 illustrates the development of the secondary response in three major lymphoid compartments in terms of the relative density of PFC compared to the spleen. It is clear that the parathymic lymph nodes always contributed a high proportion of PFC compared to the spleen as did the bone marrow at later time points.

In terms of the total number of PFC produced by the individual lymphoid compartments, the spleen certainly contributed the major proportion up until 6 days. This splenic contribution ranged from 30 to 60 per cent of the total response depending upon the day after boost, except on day 3 where it was 80 per cent and in the second and third weeks where it had dropped to 15 and 3 per cent, respectively. Between 7 and 12 days, the total organ response was fairly evenly divided among the spleen, mesenteric lymph node and bone marrow compartments. As the response progressed, the bone marrow furnished an increasingly greater proportion of the total PFC, so that at 2 and 3 weeks the contribution was 65 and 88 per cent, respectively, of the total PFC response.

Fig. 3 illustrates the development of the response in four major lymphoid compartments in terms of the total PFC contribution. While the proportion of splenic and parathymic lymph node PFC was dropping, it was rising steadily in the bone marrow.

Secondary humoral response

In Fig. 4 is shown a comparison of the total PFC response and the humoral antibody response. It can be seen that the serum and cellular antibody levels are dropping at about the same rate late in the response, so that all of the circulating antibody can be accounted for. The major source of this late serum antibody was evidently provided by the persistent maintenance of PFC by the bone marrow.

Specificity of the response

In order to study the specificity of the response, inhibition of plaque appearance was carried out using seven chemically related gallinaceous egg white lysozymes. Table 3(a) and (b) lists the percentage inhibition by each of the different lysozymes for cell suspensions derived from whole organs or from small sections of spleen, mesenteric and parathymic lymph node.

There were no unusual deviations in inhibition by hen (100 per cent), bob-white (97 per cent), duck (41 per cent), Japanese quail (93 per cent), peafowl (89 per cent), ringed-neck pheasant (55 per cent) or turkey (84 per cent) in any of these three tissues. This is consistent with a recirculation of memory cells throughout the lymphoid system and argues for a mixing of plaque-forming B memory cells.

DISCUSSION

Highly active parathymic lymph nodes

The most dramatic observation was that the parathymic lymph nodes and not the spleen contained the highest concentration of PFC throughout the primary response and during the first 2 weeks of the secondary response. In terms of the total number of PFC per animal, the spleen, mesenteric lymph nodes and bone marrow were the major contributors. PFC were also found in substantial numbers in the rest of the peripheral lymphoid organs where they appeared transitorily. Very few PFC were found in the thymus or liver. As the number of PFC was decreasing in most of the peripheral organs, they maintained a basal but significant level in the bone marrow.

The total bone marrow contribution was calculated from the PFC response of the femoral marrow cells and the estimates of Chervenick *et al.* (1968). In making this calculation, it was assumed that there was an equal distribution of PFC throughout the total bone marrow compartment of the mouse. In order to test this assumption, a comparison was made of the density of PFC found in the marrow cavities of the femoral, humeral and pelvic bones. The density of PFC was the same in all three sites (data not shown), suggesting that the assumption was valid and, therefore, the approximation of the total bone marrow contribution was fairly accurate.

Possible general control mechanisms

The high ratio of PFC found in the parathymic lymph nodes compared to the spleen has been observed in eight different inbred strains of mice. The one exception has been the SJL strain of mice in which the concentration of PFC in the spleen was slightly higher than that in the parathymic lymph nodes (D. Kipp, personal communication). It is possible to imagine that the very high rate of antibody production by the parathymic lymph node cells in many strains of mice is due to a lack of suppressor controls at this site (Gershon, 1974). The usual delicate balance between helper and suppressor activity, therefore, may be tipped towards suppression in the SJL and towards help in most other strains of mice.

Throughout the later stages of the secondary response, it was observed that there was no decay in PFC activity in the bone marrow or thymus compared to the other antibody producing sites. This constancy of the response suggests that long-lived PFC may remain sequestered there, protected from the usual regulatory control exerted by antigen, antibody, or possible suppressive mechanisms. Uncovering the causes underlying these observations should lead to an understanding of the complexities of the homeostatic mechanisms associated with maintenance of immune reactivity.

Clonal mixing of memory cells and PFC

The broad distribution of PFC activity raised the question about whether the producing cells seen in the various tissues represented recirculating cells derived from the parathymic lymph nodes and spleen, or arose *in situ* due to stimulation by migrating antigen. A distinction between these two possibilities was approached by comparing the antibody specificities of the producing cells and then relating this information to the general migratory properties of different populations of lymphocytes in the mouse. It was found that after a secondary injection of

soluble HEL, these specificity patterns were identical among the anatomically distinct spleen, parathymic and mesenteric lymph nodes. Although plaque inhibition data must be interpreted carefully, as pointed out by North and Askonas (1974), these data still indicate that there were no drastic differences in specificity of the antibody produced within the same organ or even in several distant regions of the lymphoid system. By extrapolation then, the lack of clonal distribution of specificities is consistent with the idea that memory cells have recirculated in the animal and have settled randomly throughout the lymphoid tissues.

This type of clonal mixing has been described mathematically by Lewis (1973) who related the degree of mixing between neighbouring cells growing exponentially, to the distance between the centres of the resultant clusters and to the diffusion rate of the cells. If the cells are unimpeded by the anatomical structure of the lymphoid organ and if there are no forces which tend to hold the cluster together, e.g. organizer cells, then the chances of mixing are very real. Experimentally, clonal mixing has been observed by Green (1968) who reported that PFC directed against soluble antigens were randomly distributed in the spleen and lymph nodes of guinea-pigs.

Because of these considerations, it is very probable that not only is there mixing of lymphoid cells within a particular organ, but that these cells may be free to emigrate. Once in the lymph and blood, they can recirculate and seed throughout the lymphoid system. This is supported by the evidence that long-lived lymphocytes (Röpke, Hougen and Everett, 1975; Röpke and Everett, 1974) and in particular, primed B cells (Strober and Dilley, 1973) can recirculate freely. This type of migration can occur within 24–48 h (Werdelin, McCluskey and Witebsky, 1970) and can explain the production of antibody at sites remote from the site of localization of antigen and the development of widespread secondary reactivity.

Development of systemic immunity

It was observed that after a primary injection of HEL, no significant PFC activity was detectable in a pool of the accessory axillary, brachial and inguinal lymph nodes. PFC were only demonstrable in these accessory sites after a secondary injection of HEL. Consistent with this evidence is the view that during the primary period memory cells were migrating to the accessory sites only to be expressed as PFC after a secondary soluble injection of antigen.

There is abundant evidence that PFC appear in distant lymphoid tissues after primary detection in local lymphoid organs which drain the site of injection. Daniels and Weigle (1968) have shown that the primary response to bovine serum albumin injected intravenously into rabbits occurs in the spleen, while after a secondary injection, PFC are also present in the mesenteric lymph node. This migration of memory cells was also suggested by Nash (1973) who showed that in AKR mice primed with soluble sheep erythrocyte stroma via the lower respiratory tract, there was no detectable antibody response in the spleen until a secondary intraperitoneal boost had been given. Similarly, Yowell and Hill (unpublished observations) have detected a high concentration of PFC in the regional popliteal lymph nodes of mice injected in the hind foot pads with HEL. Subsequent to a secondary intraperitoneal injection, a substantial increase in PFC activity occurred in the spleen.

Migration of immunocompetent cells and not antigen

In most of these studies, it was never determined that the appearance of PFC distal to the point of injection arose from migration and subsequent reseeding by memory cells or from migration and subsequent stimulation of local lymphocytes by antigen. However, Weissman, Peacock and Eltringham (1973) have shown that the appearance of PFC in the spleen and antibody in the serum after an injection of sheep erythrocytes into the footpads, could be inhibited by an early irradiation of the draining lymph nodes. This suggests that the presence of splenic PFC depends on a radiosensitive population of cells activated at the site of injection, and not on the spread of the antigen. Similarly, Hall, Morris, Moreno, and Bessis (1967) have interrupted the lymphatic pathway in sheep by collecting the lymph from antigen-stimulated lymph nodes, thereby preventing the development of systemic immunity.

In conclusion, it was observed that there was a change in the distribution of PFC activity from the sites immediately draining the local injection, to a generalized systemic response during the major portion of the response to the long-lasting maintenance by the bone marrow compartment during the late phases. In view of the evidence presented, it is very likely that the widespread appearance of PFC

after one or two injections of HEL is due in large part to the migration of memory cells from the primary sources.

Even though many immunological experiments performed measure the density of antibody-forming cells, the mouse parathymic lymph nodes are not usually assayed after intraperitoneal injection of protein antigens. In order to obtain maximum sensitivity, e.g. in the detection of antibody production in cases of tolerance induction or suppression, or genetically controlled immune unresponsiveness, it would seem appropriate to assess this highly concentrated source of PFC.

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