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The Morphology of Immune Reactions in Normal, Thymectomized and Reconstituted Mice

I. THE RESPONSE TO SHEEP ERYTHROCYTES

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Summary. The response to sheep red blood cells has been studied in the lymph nodes draining their site of injection in normal mice, and in thymectomized, irradiated, bone-marrow injected mice with and without a reconstituting thymus graft. By using a chromosome marker to differentiate between cells derived from the bone-marrow and thymus graft it has proved possible to show that the immune response should be thought of in terms of at least two cell populations. Cells of thymic origin are stimulated to mitotic activity in the interfollicular cortex, and their activity precedes both antibody production and morphological signs of activity in the follicular regions. Mitotic divisions of cells of bone-marrow origin reached a peak a day later than did the thymic cells and their activity was sustained. Follicular enlargement and germinal centre production were coincident in time both with antibody production and bone-marrow cell mitotic activity. Lymph nodes of animals lacking a thymic influence showed only minor changes after antigenic stimulation and these were restricted to the follicular regions. There appeared to be only a small quantitative difference between the responses of normal and of reconstituted animals.

INTRODUCTION

Recovery of immunological responsiveness in irradiated adult mice has been shown to depend to some extent on the presence of a functioning thymus. This conclusion derives from two complementary types of experiment. Firstly, animals deprived surgically of their thymus prior to irradiation, with or without subsequent injection of bone-marrow cells as a therapeutic measure, show a sustained impairment of response to certain antigenic stimuli (Miller, 1962; Miller, Doak and Cross, 1963); sham-thymectomized controls show progressive recovery of responsiveness. Secondly, a thymus graft can effectively reconstitute the thymectomized irradiated animal in such a way that immunological competence returns after irradiation (Leuchars, Cross and Dukor, 1965). In this second type of experiment, it seems that the lymphoid cells which are released from the grafts play an important role in the immunological restoration; they show a mitotic response to various antigenic stimuli (Davies, Leuchars, Wallis and Koller, 1966), but apparently do not produce antibody (Davies, Leuchars, Wallis, Marchant and Elliott, 1967). These thymus-derived cells have been termed reactor cells (Gershon, Wallis, Davies and Leuchars,

1968) and the recognition that they are distinct from antibody-producing effector cells may be a useful step in clarifying the cellular basis of the immune response. However, the present experimental evidence relating to them is insufficient for any satisfactory generalization to be made. It was, therefore, decided to make a detailed study of reactor cell responsiveness to a variety of antigenic stimuli.

Immune responses have been classified in a number of ways: in the present context it is useful to consider three types. In mice, local application of skin-sensitizing agents, such as 2-phenyl-4-ethoxymethylene-5-oxazolone (oxazolone), induces initially a state of cell-mediated immunity accompanied by marked hyperplasia of paracortical cells in the draining lymph nodes (Oort and Turk, 1965; Turk, 1967). Injection of immunogenic doses of antigens such as pneumococcal polysaccharide leads to the production of humoral antibody and proliferation of cells in germinal centres and medullary cords (Parrott and de Sousa, 1966). Heterologous erythrocytes injected into mice elicit hyperplasia in both the paracortical regions and in germinal centres. Each of these three types of antigen has been used to elicit a response in the lymph nodes of mice whose immunological competence depended upon the presence of a cytologically-marked thymus graft. Studies have been made of histological changes, reactor cell activity and, where appropriate, antibody formation following a single primary contact with antigen. The response to sheep red blood cells is reported here.

Previous work on reconstituted mice concerned the response in the spleen to intraperitoneal injections of sheep red cells (Davies *et al.*, 1966). Although this gave a clear demonstration of thymus-derived cell activation it was difficult to determine the location of the activated cells and also the magnitude of the response. The choice in the present experiments of oligosynthetic (Olson and Yoffey, 1966), draining lymph nodes was in part intended to remedy these defects and also to provide organs in which the responses to a wide variety of stimuli could be compared.

MATERIALS AND METHODS

CBA/H male mice were thymectomized at 8 weeks of age. Two weeks later, they were subjected to 850 r total body irradiation from a 220 kV X-ray machine. Within 3 hours, an intravenous injection of 5×10^6 cells of syngeneic (CBA/H) bone-marrow was given. These mice were then grafted with a single lobe of a CBA/H-*T6T6* neonatal thymus which was implanted under the capsule of the left kidney. Approximately 120 animals were prepared in this way and they will be referred to as *reconstituted* mice. A further sixty mice, which were not given thymus grafts, will be called *deprived.* A group of sixty *normal* male mice of the CBA/H strain was also studied. The method of preparation of the chimaeras has been published before, Davies *et al.* (1966).

Two experimental procedures were undertaken:

(1) *Cytology*

Reconstituted mice, four in a box, were left until 50 days after irradiation and implantation of the thymus grafts. Each of three mice in every box was then injected with 5×10^8 sheep red blood cells in 0·2 ml of Alsever's solution. The inoculum was divided equally between four sites—the two forefeet and two sites high up on the back. The intention was to stimulate a reaction in the internal and external axillary lymph nodes by intradermal or subcutaneous injection. Every day for 10 days following these injections, a box

of mice was taken and the axillary lymph nodes were removed for cytological analysis, the uninjected mouse in each box serving as a control. Each lymph node was dealt with separately so that about twelve experimental and four control nodes were examined every day. The manner of preparation of cells for cytological analysis was basically the same as that described by Ford (1966). The slides from each node were coded and inspected independently by different workers. Distinction was made, as before, between CBA/H-*T6T6* cells derived from the thymus graft and CBA/H cells derived from the bone-marrow inoculum, Davies *et al.* (1967). Where possible, fifty dividing cells were scored on each slide.

(2) *Histopathology and serology*

Reconstituted, deprived and normal mice were placed five in a box. Fifty days after irradiation, five mice in each group were weighed and killed. Axillary lymph nodes and thymus tissue (if present) were removed, weighed, and fixed in Bouin's solution. Paraffin sections were prepared at 5 μ and stained with haematoxylin and eosin and, in some instances, with Giemsa and by Gordon and Sweets' silver impregnation technique for reticulin fibres. All sections were coded and examined independently. All but fifteen of the remaining mice were injected with sheep red blood cells as above. On each of the 10 subsequent days, serum samples were taken from five mice in each group before they were killed.

Sera were titrated for anti-sheep-red-cell antibodies in saline, either as haemagglutinins or haemolysins. On the 11th day, the fifteen remaining mice were killed and their axillary lymph nodes were fixed. Thus, control mice were examined at both the beginning and the end of the experiment.

RESULTS

SEROLOGY

The agglutinin titres of the three different groups of mice are recorded in Fig. 1; the lysin titres are not included as they were similar to the agglutinin titres. It is clear that the amount of antigen given is effectively immunogenic in normal mice. The reconstituted mice, though responding less vigorously than normal, showed a similar titration response. Two conclusions emerge: firstly, reconstituted mice are not as immunologically responsive as normal mice; secondly, recovery of the immune response to sheep red cells after irradiation is partly thymus-dependent.

PATHOLOGY

The lymph node, thymus and body weights of the experimental animals are recorded in Table 1. It will be noted that normal mice are heavier than reconstituted and deprived mice. After injection of antigen, there was a slight drop in body weight in normal mice but a considerable reduction occurred in the body weight of reconstituted and deprived animals, lasting for 3 days. No obvious changes in the weights of either the thymuses in normal mice or of the thymus grafts in the reconstituted animals were observed. The normal mice had approximately twice as much thymus tissue as the reconstituted mice. The lymph node–body weight ratios (Fig. 2) show a pronounced increase in normal and

TABLE 1

LYMPH NODE AND THYMUS WEIGHTS (AS RATIOS OF BODY WEIGHTS), AND BODY WEIGHTS OF NORMAL, DEPRIVED AND RECONSTITUTED MICE AT VARIOUS TIMES AFTER LOCAL INJECTION OF SHEEP RED BLOOD CELLS (CONTROL MICE ARE NOT INJECTED)

	Starting control	Injected mice (time in days after injection)										Finishing control
		1	2	3	4	5	6	7	8	9	10	
Body weight (g) ($n = 5$)	34·6	34·6	31·6	35·0	36·0	36·0	34·8	34·4	34·0	34·2	33·6	35·2
1. Normal	± 1·8	± 3·6	± 3·2	± 6·8	± 2·9	± 2·7	± 1·5	± 2·5	± 2·3	± 2·9	± 1·7	± 2·9
2. Deprived	25·8	26·8	25·2	23·4	25·0	27·0	25·4	22·3	25·0	25·4	21·2	23·8
	± 2·6	± 1·3	± 2·6	± 1·7	± 2·2	± 1·9	± 1·1	± 1·5	± 1·0	± 1·8	± 1·5	± 1·5
3. Reconstituted	29·4	30·0	25·2	24·0	24·8	29·7	25·7	30·2	25·0	27·4	24·7	26·7
	± 0·9	± 2·9	± 4·8	± 1·0	± 0·8	± 0·5	± 1·2	± 4·2	± 0·7	± 2·3	± 2·6	± 2·2
$\frac{\text{Thymus weight}}{\text{Body weight}} \times 10^4$ ($n = 5$)	7·94	7·71	6·85	6·53	7·05	7·72	7·09	7·72	8·58	7·58	7·77	7·49
1. Normal	± 0·77	± 0·84	± 0·74	± 0·93	± 1·49	± 0·55	± 0·33	± 0·73	± 1·33	± 0·36	± 0·82	± 0·82
2. Reconstituted	3·67	2·84	4·76	3·70	3·33	4·16	4·15	2·64	3·57	4·22	4·46	3·76
	± 0·80	± 0·22	± 1·70	± 0·32	± 0·66	± 0·23	± 0·99	± 0·42	± 0·33	± 0·45	± 1·00	± 0·33
$\frac{\text{External axillary lymph node weight}}{\text{Body weight}} \times 10^4$ ($n = 10$)	0·82	1·35	2·45	2·51	3·01	3·73	2·83	2·07	2·05	2·16	2·08	1·28
1. Normal	± 0·12	± 0·32	± 0·57	± 0·56	± 0·50	± 1·19	± 0·83	± 0·40	± 0·39	± 0·34	± 0·38	± 0·18
2. Deprived	0·63	0·57	1·13	0·74	0·82	0·72	0·92	0·59	0·62	0·78	0·84	0·91
	± 0·20	± 0·22	± 0·20	± 0·28	± 0·27	± 0·17	± 0·13	± 0·17	± 0·23	± 0·27	± 0·24	± 0·30
3. Reconstituted	0·79	1·05	2·17	2·28	1·86	2·46	2·26	1·14	1·76	1·49	1·73	1·18
	± 0·17	± 0·25	± 0·55	± 0·28	± 0·62	± 0·26	± 0·42	± 0·17	± 0·34	± 0·18	± 0·28	± 0·29

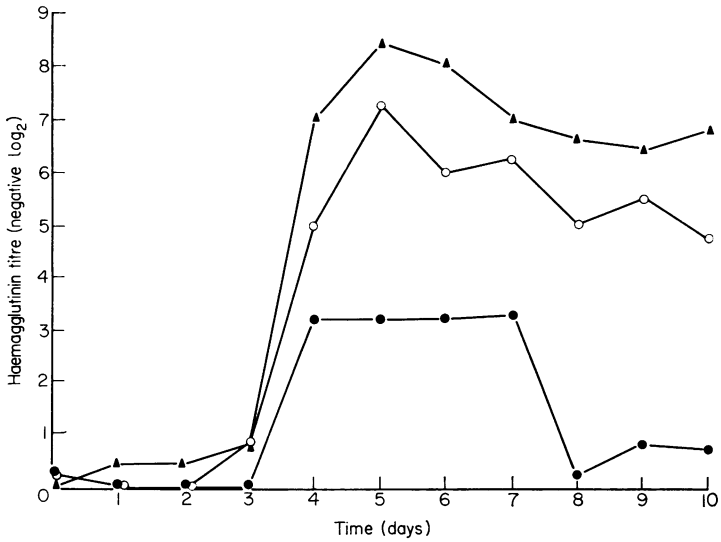

FIG. 1. The haemagglutinin titres of normal (▲), deprived (●) and reconstituted (○) mice (see text) in relation to time after administration of a single injection of 5×10^8 sheep red blood cells.

reconstituted animals but, in deprived mice, there was only a slight upward change. The internal axillary nodes responded in a similar manner to the external and are not, therefore, included in the results.

Four hundred and twenty-six axillary lymph nodes were examined from the three test groups—153 from normal mice, 149 from deprived mice and 124 from reconstituted

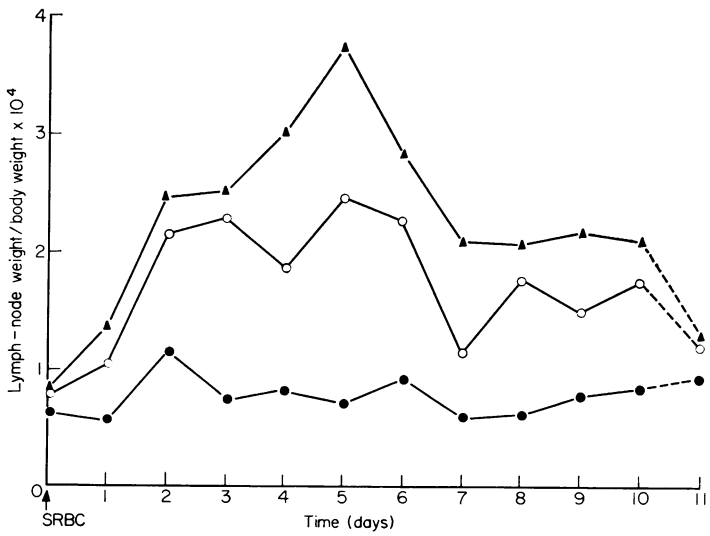

FIG. 2. Mean external axillary lymph node weights of normal (▲), deprived (●) and reconstituted (○) mice (see text) plotted as proportions of body weight at various times after local injection of sheep red blood cells.

animals. A further eighty-four axillary lymph nodes were examined from twenty-nine uninjected mice. A well-defined morphological response developed in the three test groups although stimulation of all the axillary nodes, at any one time, was never observed; on each of the 10 days of the experiment, a variable proportion of the nodes examined was morphologically inactive.

Uninjected mice

Small numbers of histiocytes and lymphoid cells were usually present in the superficial sinuses but the deeper parts of the pulp appeared to be quiescent: no active follicles were seen, the paracortical area was composed of mature lymphoid cells, and the medullary cords were inconspicuous.

Normal mice

Initially the nodes were normal and appearances were similar to those described in uninjected mice. One day after injection of sheep erythrocytes, a moderate increase was noted among immature lymphoid cells in the paracortical area (Fig. 3). The cells were big blast-like elements with a large vesicular nucleus, a prominent nucleolus, and a thin rim of basophilic cytoplasm; mitotic figures were sometimes seen. These cells increased in number until the 4th and 5th days and then declined. Follicular enlargement was first apparent on the 3rd day after injection of sheep cells. Initially, the follicles formed small compact structures ranged round the edge of the node. Later, they increased in number and size but still retained their peripheral distribution. The germinal centres (Fig. 4) consisted of a core of large basophilic blast cells, among which mitotic figures were numerous. The surrounding cuff of small lymphocytes was poorly demarcated and, in the later stages, coalescence of adjacent germinal centres was sometimes noted. The number of cells in mitosis declined at around the 8th day: pyknotic lymphoid cells and amorphous cell debris became more prominent in the germinal centres and some of this material was engulfed by invading histiocytes. Moderate enlargement of the medullary cords (Fig. 5) occurred at the same time as the follicular changes. Enlarged cords extended into the pulp and were seen to be composed of a variety of cell types including blast cells and immature plasmacytic elements. The changes in the follicles and medullary cords were sustained and considerable activity was still apparent (particularly in the follicles) at the end of the experiment, 10 days after the injection of sheep erythrocytes.

Deprived mice

Axillary lymph nodes in deprived mice differed strikingly from the other nodes which were studied by the presence of a hypoplastic paracortical area which persisted unchanged throughout the experiment. The normal components, consisting of dense sheets of lymphocytes, were replaced by large mononuclear cells with abundant eosinophilic cytoplasm which were thought to be histiocytes. A few small lymphocytes were seen but large blast cells similar to those previously encountered in normal mice were not observed at any time. The underlying reticulin framework was intact and the individual fibres appeared to be thickened. Despite the striking hypoplasia of the paracortex, reactive changes developed in the follicles on the 3rd day after injection (Fig. 6). The subsequent follicular response was comparable to that described in normal mice except that the follicles were fewer and smaller and their phase of activity lasted for a shorter period of

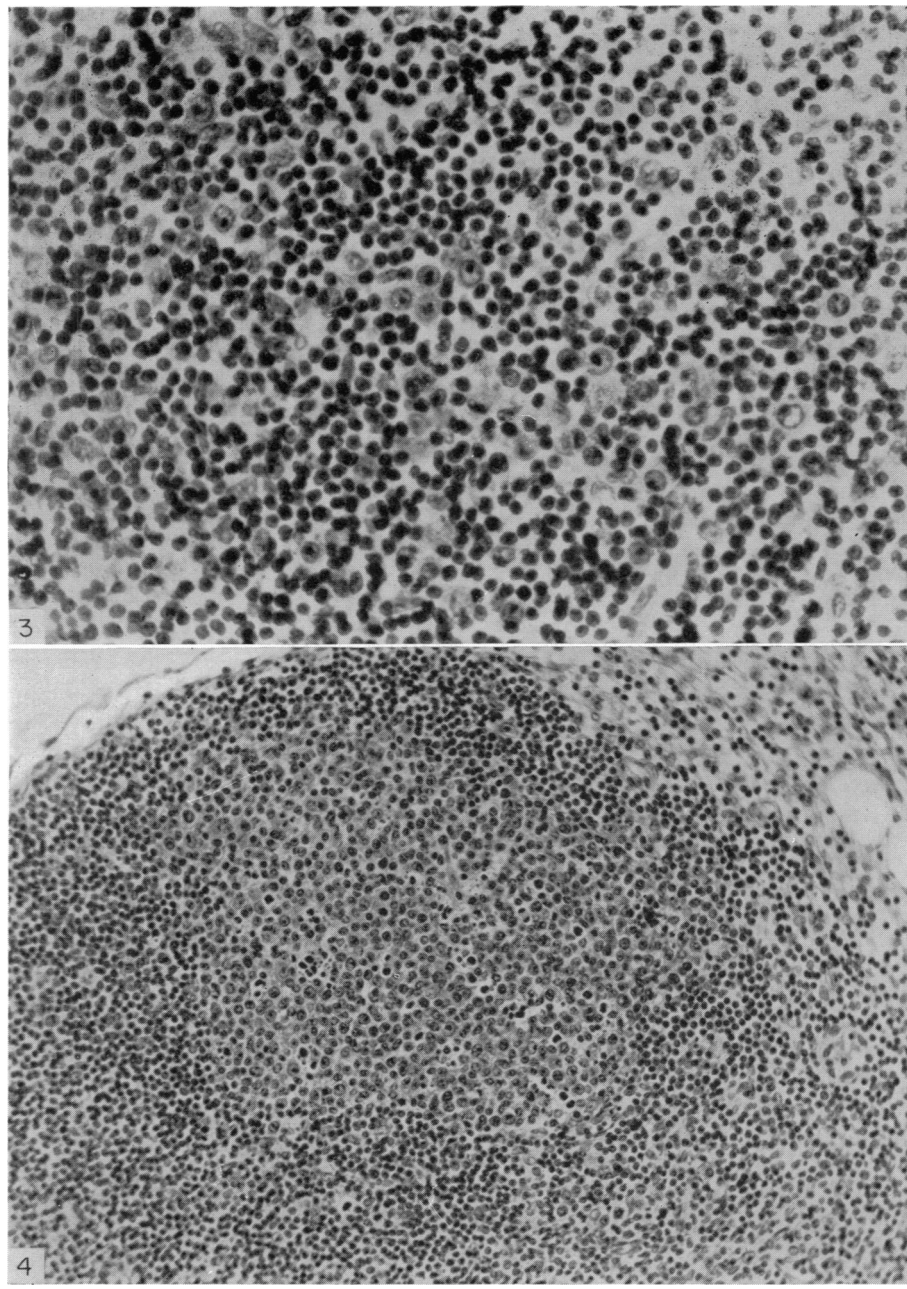

FIG. 3. Normal mouse, 3 days after injection of sheep cells; axillary lymph node. There are increased numbers of immature lymphoid cells in the paracortex. H & E, ×460.

FIG. 4. Normal mouse, 6 days after injection of sheep cells; axillary lymph node. Primary follicle with well-developed germinal centre. H & E, ×240.

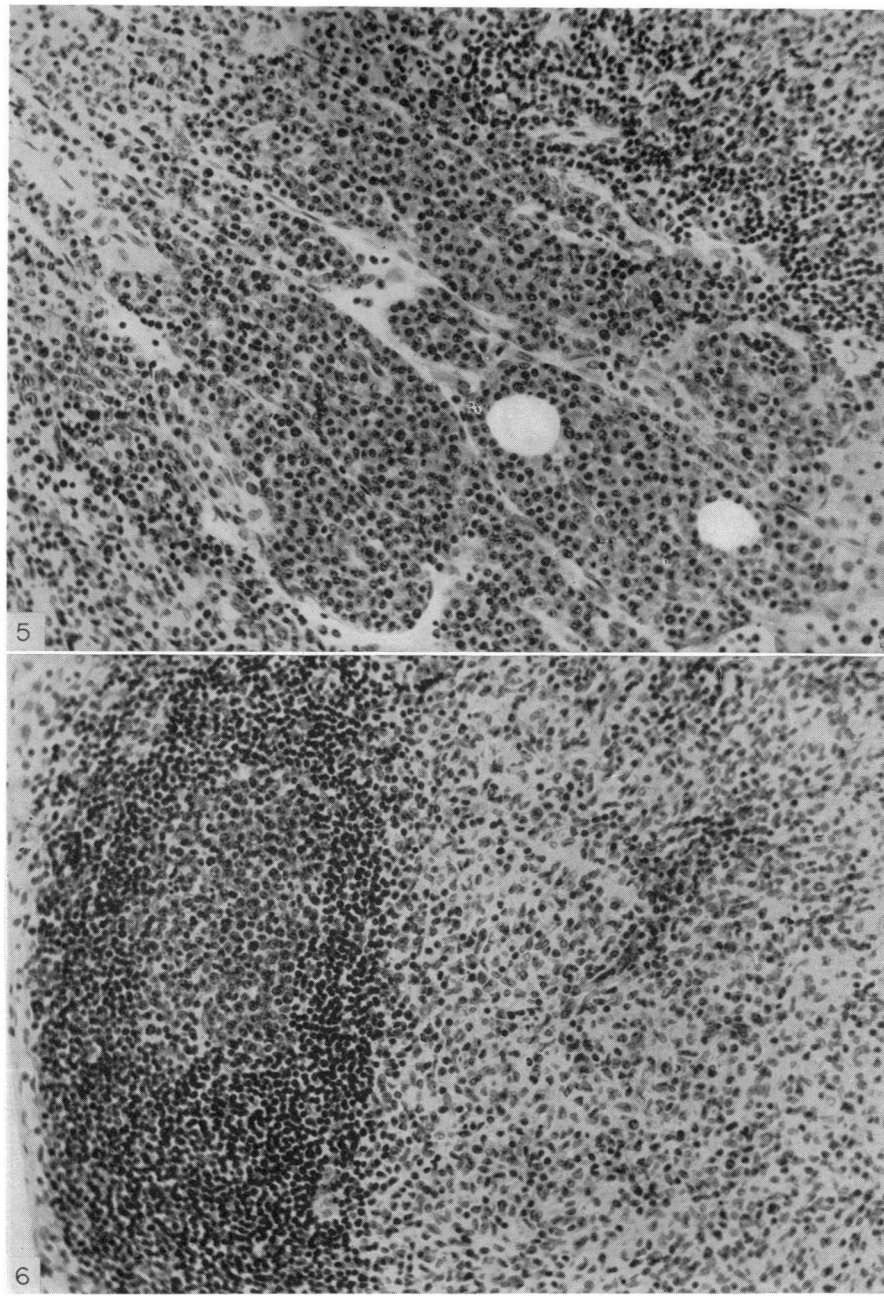

FIG. 5. Normal mouse, 7 days after injection of sheep cells; axillary lymph node. There is moderate hyperplasia of the medullary cords. H & E, ×240.

FIG. 6. Deprived mouse, 5 days after injection of sheep cells; axillary lymph node. Slight activation of germinal centres (cf. Fig. 4). The hypocellular paracortical area is also apparent. H & E, ×240.

time. Changes in the medullary cords showed a similar time course, with enlargement on the 3rd day which then quickly subsided.

Reconstituted mice

The morphological response encountered in nodes from reconstituted mice was similar to, though less marked than, the changes described in normal animals. Appearances were initially normal but some hyperplasia of paracortical blast cells was seen on the second day, 24 hours before enlargement of follicles and medullary cords became apparent. Considerable activity was later seen in these structures, particularly in the follicles, but the changes were less sustained than in nodes examined at a comparable time from normal mice. By 10 days, the response was clearly waning. In some mice, the medullary cords were noticed to be somewhat hypocellular at the later stages of the experiment.

Three conclusions emerge from this part of the experiment. Firstly, injection of sheep red blood cells into normal CBA/H mice leads to a series of changes in the regional lymph nodes which include early activation of the paracortical regions, stimulation of the follicles with germinal centre formation, and moderate enlargement of the medullary cords. Secondly, lymph nodes from reconstituted mice were found to behave similarly but less vigorously. Thirdly, lymph nodes from deprived animals show no paracortical activity while reactive changes in the follicles and medullary cords are diminished and of short duration.

CYTOLOGY

The results of the cytological analysis are given in Table 2 and Fig. 7. The values obtained for individual lymph nodes are not given as they varied little at the times of peak reactivity in injected mice. The use (in Fig. 7) of the average number of cells scored of a

TABLE 2

CYTOLOGICAL ANALYSIS OF THE AXILLARY LYMPH NODES OF RECONSTITUTED MICE AT VARIOUS TIMES AFTER LOCAL INJECTION OF SHEEP RED BLOOD CELLS

Mice	Days after injection	No. of LNs examined	Total No. of cells scored	No. of thymus-derived cells	Per cent of thymus-derived cells
Injected	1	12	6	2	33·3
	2	10	162	82	50·6
	3	12	664	396	59·6
	4	12	625	144	23·0
	5	12	207	24	11·6
	6	12	178	23	12·9
	7	12	59	8	13·6
	8	12	48	3	6·2
	9	12	67	7	10·4
	10	8	29	7	24·1
Uninjected		26	88	28	31·8

particular chromosomal phenotype per lymph node underestimates, in some cases, the real numbers of such cells per node; small nodes yielded only sufficient cells to make one or, at the most, two slides. Larger nodes, such as those found on days 3, 4, 5 and 6, gave enough cells to make six or seven slides but only two were made. Thus the analysis of unreactive nodes involved inspection of all available cells but from reactive nodes only a sample was taken.

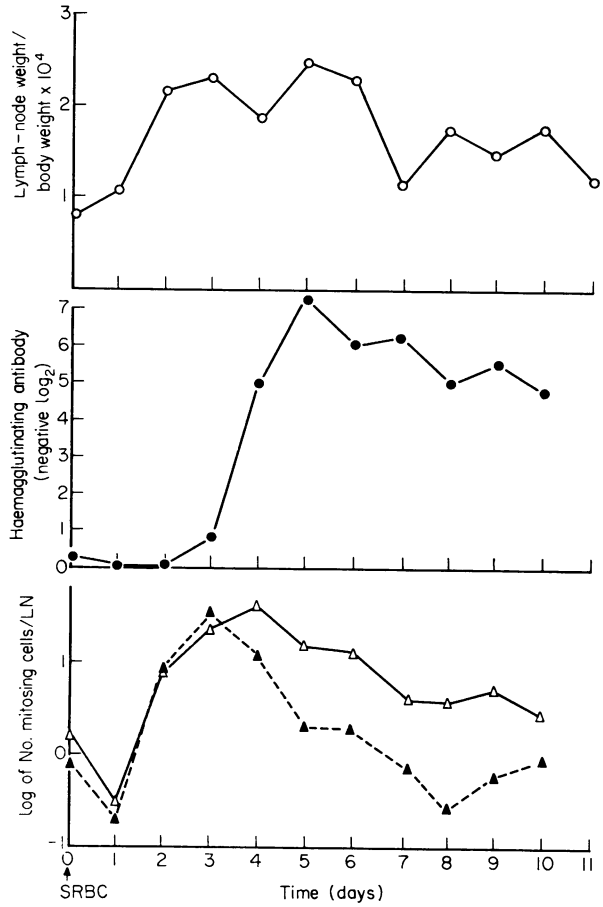

FIG. 7. A comparison of changes in lymph node/body weight ratios, antibody titre and mitotic activity over a 10-day period after local injection of sheep red blood cells into reconstituted mice. △, Bone-marrow derived; ▲, thymus derived.

The day by day values for control nodes are not given as they showed no meaningful fluctuations. Their principal use was to demonstrate that mitoses were rare in the nodes of control mice. Dividing cells in control nodes were of both thymus and bone-marrow origin in the ratio 1:2.

After injection of sheep cells it can be seen that an increase in the numbers of cells in mitosis occurred and that first the thymus- and then the bone-marrow-derived cells were stimulated. Augmented thymus-derived cell mitosis was not evident by the 5th day but bone-marrow-derived cells showed mitotic activity above normal until about the 10th day. The proportions of the two cell types are shown in Fig. 7. For a short time there are more thymus-derived than bone-marrow-derived cells in mitosis.

It can be concluded that the mitotic events in draining lymph nodes after injection of antigen should be considered in terms of two cell populations—thymus- and bone-marrow-derived—which reach their peak mitotic activities in 3 and 4 days, respectively.

These various parameters of the immune response after injection of sheep cells can be

correlated in time (Figs. 7 and 8) as follows. In *normal* mice, the earliest change seen in the draining nodes was an increase in the numbers of large blast-like cells in the paracortical zones. This occurred before antibody was detectable in the serum and was associated with substantial enlargement of the node. Antibody was first recorded on the 4th day, following an increase in follicular and medullary cord activity. Soon afterwards, the activity in the paracortical zone subsided. The lymph nodes continued to gain weight until the 5th day and were heavier than normal for the rest of the experiment. Follicular activity remained high. The findings in *reconstituted* mice were broadly similar to those in normal animals but their responses seemed in all respects less vigorous. In these animals, the additional parameter of thymus-derived cells could be studied. The mitotic activity of these cells coincided in time with the heightened activity observed in the paracortical zone and also

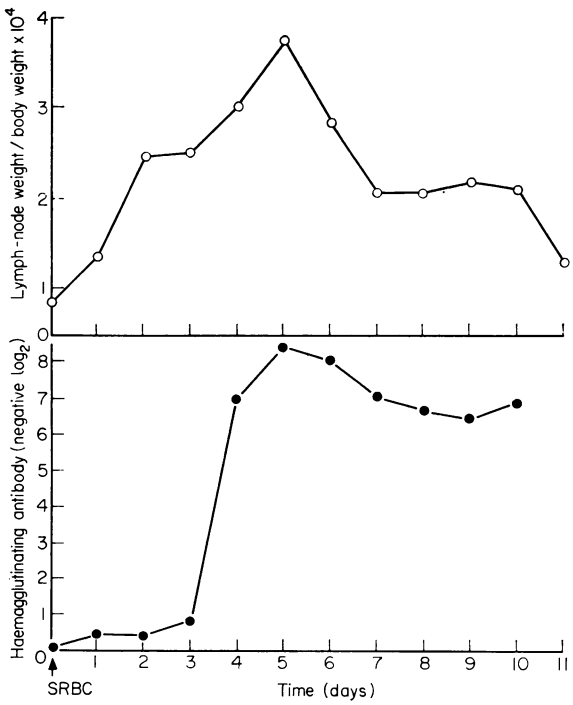

FIG. 8. A comparison of changes in lymph node/body weight ratios and antibody titre over a 10-day period after local injection of sheep red blood cells into normal mice.

with the increase in lymph node weight on day 3. It is noteworthy that the earliest weight increase in the lymph nodes of reconstituted mice preceded an increase in mitotic activity of either bone-marrow or thymus-derived cells. High mitotic activity of cells of bone-marrow origin preceded the peak lymph node weight and coincided with the period of rapid increase in antibody titre. It is not possible to say that bone-marrow-derived cells in mitosis at this peak of activity were located in either paracortical or follicular regions

since mitoses were observed in both these regions on day 4. Later, mitotic activity of cells of bone-marrow origin was likely to have been almost entirely follicular. The close similarity between the findings from normal and reconstituted mice suggests that the latter are useful experimental models for evaluating immunological mechanisms in intact animals. By contrast, the *deprived* animals showed little activity after administration of antigen. It is significant that no activation of cells in the paracortical regions could be observed whereas follicular activity, though reduced and temporary, was evident.

DISCUSSION

The purpose of these experiments was to obtain several descriptions of the immune response of mice to sheep red cells. As expected, both early activation of the interfollicular cortex and subsequent heightening of follicular activity were seen. Our studies show that the principal mitotic components of the early activity were of thymic origin and that this phase of the immune response preceded detectable production of antibody. Follicular activity, which involved bone-marrow-derived cell mitoses, was associated in time with antibody production. This interpretation accords with that from other experiments which has suggested that thymus-derived cells are not antibody producing but that cells of bone-marrow origin may be involved in this process (Davies *et al.*, 1967; Miller, 1967). In addition, the association of thymus-derived cell mitosis with the paracortical area confirms Parrott's delineation of this region as thymus-dependent (Parrot, de Sousa and East, 1966; Parrott and de Sousa, 1966).

It has been postulated by a number of workers that cells from thymus and bone marrow react synergistically during the course of an immune response (Claman, Chaperon and Triplett, 1966; Davies *et al.*, 1967; Miller, 1967). Nothing is yet known of the site of this reaction nor of its nature. On the basis of the present work it might seem that the site of the reaction could be the interfollicular cortex but that its consequences are enacted in the follicular regions. Clearly, much remains to be studied here. Another problem emerges when attempts are made to compare the sites of antigen deposition with the sites in which are observed the first histological evidence of increased activity following antigen administration. Although sheep red blood cells have not been traced accurately into draining lymph nodes their distribution is almost certainly in macrophages of the marginal sinuses, the medulla and the follicular region. But the first histological signs of activity were in the paracortical area.

It would seem that we are at the beginning of an understanding of the complex sequence of events which follows administration of antigen. We feel that the use, in the first instance, of reconstituted mice may well help in this process.

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