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The Kinetics and Morphology of the Rosette-forming Cell Response in the Popliteal Lymph Nodes of Rats

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Summary. A modified ICA technique was used to study the kinetics and morphology of the RFC response in rat popliteal lymph nodes after an inoculation of SRBC into the hind footpad. The primary response was followed over a 10-day period. RFC were classified as either macrophages, haemocytoblasts, plasmacytes or lymphocytes.

RFC present in the popliteal lymph nodes of uninoculated rats were identified as macrophages and lymphocytes. After inoculation the number of RFC rose rapidly to reach a peak at 5–6 days. It was shown that after incubation at 37° certain RFC from inoculated rats had several layers of adherent SRBC and it was suggested that this was an indication of an active secretion of haemagglutinin. 3–4 days after innoculation large mature haemocytoblasts were actively secreting haemagglutinin whilst from the 5th to the 10th day plasmacytes were the RFC involved in the haemagglutinin production. It is suggested that the large haemagglutinin producing haemocytoblasts arise without mitosis via a process of cell transformation and that RF plasmacytes arise via lymphocyte activation into small haemocytoblasts, mitotic division and eventual maturation into immature plasmacytes. RF lymphocytes were thought not to be involved to any extent in haemagglutinin production.

INTRODUCTION

The phenomenon of immunocyto-adherence (ICA) can be defined as the adherence of erythrocytes around a nucleated cell due to the binding of antigens on the surface of the erythrocyte to receptor sites on the surface of the nucleated cell. These latter cells are here referred to as rosette-forming cells (RFC). The ICA technique has been extensively used in studies on the kinetics of the cellular immune response (Shearer, Cudkowicz, Connell and Priore, 1968; Storb and Weiser, 1968; Zaalberg, Van der Meul and Van Twisk, 1968) but there is a lack of information as to the morphology of RFC. Electron microscopy has been used to examine RFC (Storb, Chambers, Storb and Weiser, 1967; Storb, Bauer, Storb, Fliedner and Weiser, 1969); however the techniques involved limit the number of RFC that can be examined. In a previous paper we described a technique which enabled an accurate differential assessment of RFC to be made by light microscopy (Duffus and Allan, 1969).

There have been many reports on the cellular changes in draining lymph nodes after inoculation with heterologous erythrocytes (Eidinger, 1968; Davies, Carter, Leuchars,

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Wallis and Koller, 1969) and several reports on the cellular kinetics in such an experimental system (Eidinger and Pross, 1967; Biozzi, Binaghi, Stiffel and Mouton, 1969; McConnell, Munro, Gurner and Coombs, 1969). In the present study utilizing our modified ICA technique we attempted to follow both the kinetics and morphology of the cellular immune response in rat lymph nodes after an inoculation of sheep red blood cells (SRBC) into the hindleg footpad.

MATERIALS AND METHODS

Experimental animals

A line-bred strain of 5–6-month-old Carworth Wistar rats of both sexes were used. The weight of the rats was between 250 and 325 g.

Antigen

Sheep red blood cells (SRBC) were obtained by venopuncture from a Clun Forest ewe aged 2 years. The blood was heparinized and stored at 4° and used within 5 days. Before use the SRBC were washed six times in 0·85 per cent sterile saline.

Medium

For all experiments cells were suspended in a solution of Parkers 199 T.C. medium containing 10 per cent foetal calf serum (Difco).

Experimental plan

Thirty male and thirty female rats were injected with $2{\cdot}5 \times 10^9$ SRBC into the left hind footpad under light ether anaesthesia. A further six male and six female rats served as uninoculated controls. The primary response was followed by killing six rats for each post-inoculation day over a 10-day period. The popliteal lymph nodes were removed from the left hindleg and immediately placed in sterile medium. Blood was taken from each rat, allowed to clot at room temperature and then kept overnight at 4°. The sera were collected, heated at 56° for 20 minutes and then stored at −20°.

Lymph node cell suspension

The lymph nodes were teased apart and then further dispersed through a fine pasteur pipette. The cell suspension was filtered through a stainless steel mesh of 120 μ diameter to remove large tissue clumps and again through a fine stainless steel gauze filter of 35 μ diameter (Begg and Cousland). This filtration produced a suspension consisting almost entirely of single cells. The cell suspension was then washed three times in medium by centrifuging at 80 ***g*** for 5 minutes in siliconized glass tubes. The cells were finally resuspended in fresh medium.

ICA technique

Freshly washed SRBC were suspended in medium to give a concentration of 3×10^8 erythrocytes/ml. The lymph node cell suspension was adjusted to give a concentration of 1×10^7 cells/ml. 0·25 ml of the SRBC suspension was added to 0·25 ml of lymph node cell suspension. This gave a ratio of erythrocytes to lymph node cells of 30:1. The cell mixture was then incubated at 37° in a waterbath with gentle agitation every 15 minutes. After 2 hours incubation samples were withdrawn, diluted 1 in 20 in medium and the number of

rosettes present in 5×10^4 lymph node cells was counted in Improved Neubauer haemocytometers. A white cell count was performed on each sample and the number of rosette forming cells (RFC)/1000 lymph node cells was calculated. Cells with five or more adherent SRBC were scored as RFC.

Preparation of permanent films

To examine the morphology of RFC, samples of the incubated cell suspensions were centrifuged on a cytocentrifuge (Duffus and Allan, 1969). The slides were dried in air, stained with triple Leishman and mounted. These were later examined and a differential cell count was performed on fifty to 200 RFC from each rat. However, in certain of the uninoculated control rats the low count of RFC reduced this number to between twenty and fifty RFC.

Direct haemagglutination tests

Serial two-fold dilutions of the serum samples were made in 0·2 ml of saline. To each tube 0·2 ml of a 1 per cent suspension of washed SRBC was added and after incubation for 1 hour at 37° the haemagglutinin titres were recorded.

RESULTS

KINETICS OF IMMUNE RESPONSE

Direct haemagglutinin titres were determined for each serum sample from the twelve control rats and the sixty inoculated rats. The results are shown in Fig. 1. In none of the serum samples from control rats were haemagglutinin titres demonstrated. The inductive period for the appearance of serum haemagglutinins was 24–48 hours, followed by a rapid increase over the next 4–5 days, then by a more gradual increase still evident 10 days after inoculation. This pattern of haemagglutinin production is comparable to that described by Eidinger and Pross (1967) for a similar SRBC/footpad inoculation system.

The number of RFC/1000 lymph node cells was calculated for all control and inoculated rats. The results are shown in Fig. 2. The average RFC count for the twelve control rats was 0·67 RFC/1000 cells. The count for the six male rats was 0·4 RFC/1000 cells and for the six female rats was 0·92 RFC/1000 cells; this difference proved significant ($P<0·001$). Amongst the inoculated rats there was no significant difference in the response of males and females. 24 hours after inoculation the count had risen to an average value of 1·9 RFC/1000 lymph node cells. After 48 hours the count had dropped to 1·25 RFC/1000 and then increased rapidly to reach a peak of 14·3 RFC/1000 5 days after inoculation. The number of RFC then fell gradually and at the tenth day the average count was 6·1 RFC/1000, still considerably above that found in the uninoculated control rats.

Using the technique described above for SRBC aliquots of lymph node suspensions from each of five inoculated rats were mixed with homologous erythrocytes. No RFC were observed.

APPEARANCE OF RFC IN WET PREPARATIONS

Examples of rosettes from wet preparations are shown in Figs 3(A–F). Incubation at 37° resulted in a variation in the size of rosettes and this finding is clearly illustrated in Figs 3(A and B). In order to reveal the central RFC in such preparations the coverslip was

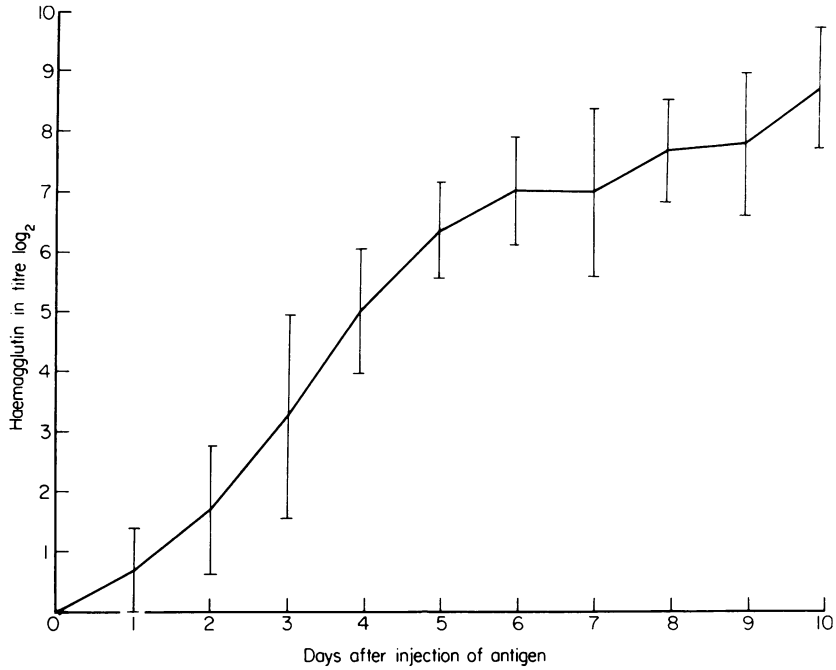

FIG. 1. Average serum haemagglutinin response before (day 0) and following injection of SRBC. The value for day 0 was determined from twelve control rats and for each subsequent day from six inoculated rats. The vertical lines indicate standard deviation from the mean.

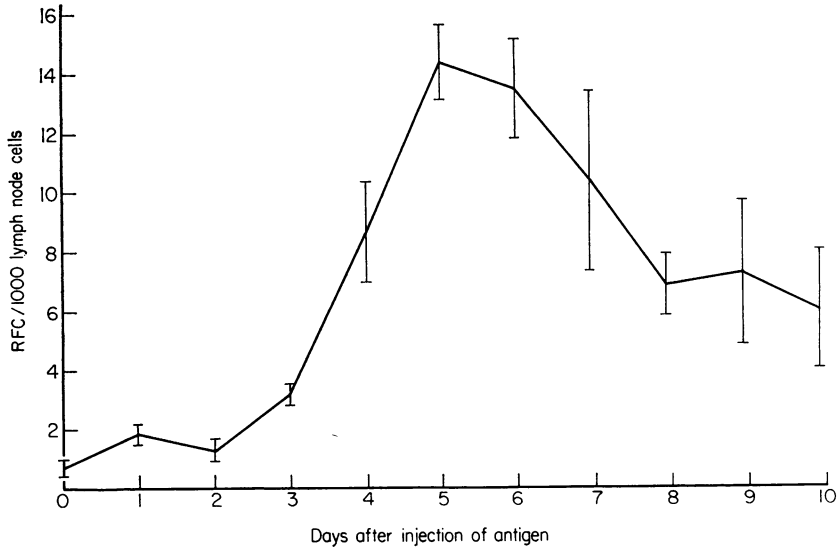

FIG. 2. Average number of RFC/1000 lymph node cells before (day 0) and following injection of SRBC. The value for day 0 was determined from twelve control rats and for each subsequent day from six inoculated rats. The vertical lines indicate standard deviation from the mean.

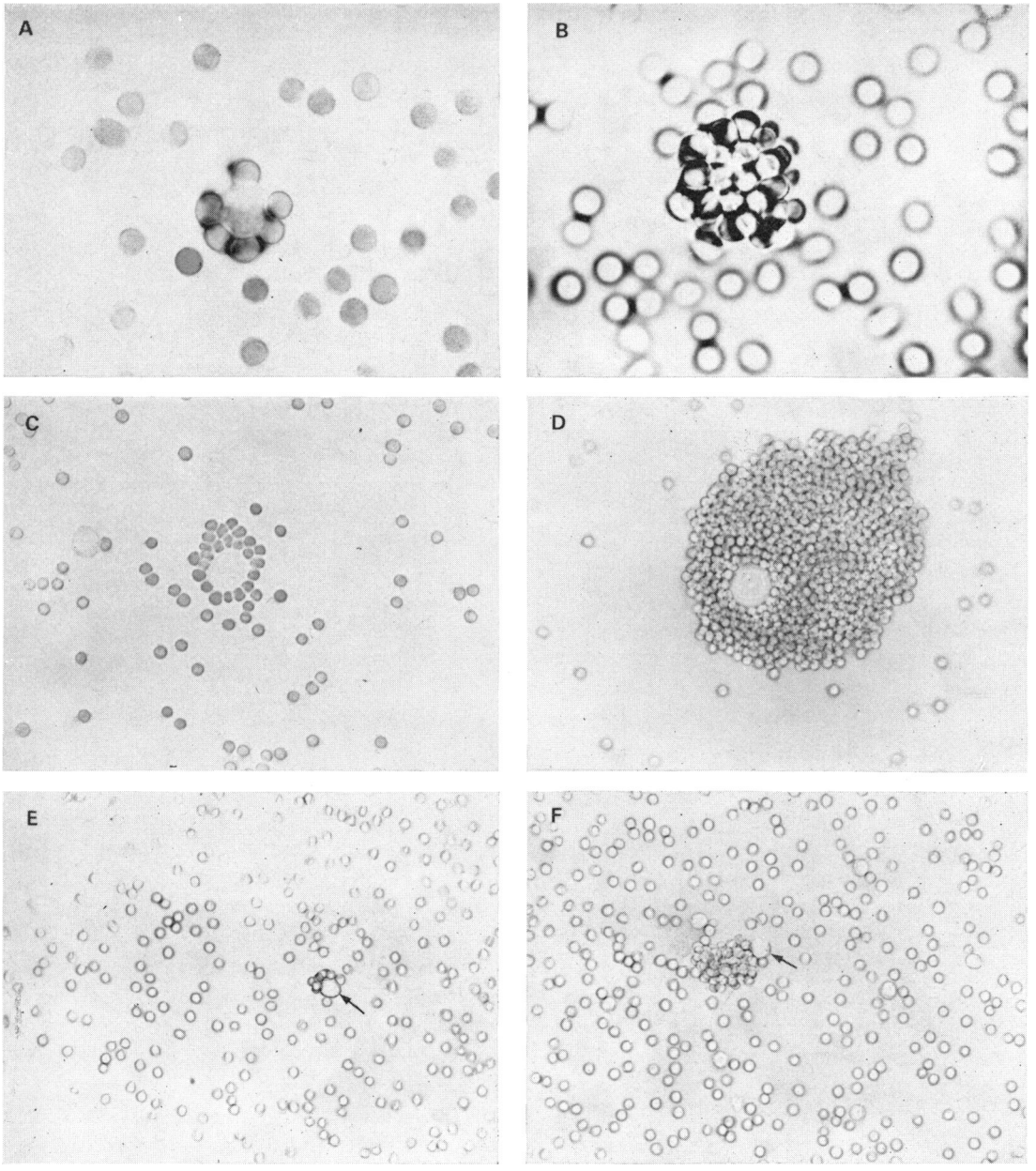

FIG. 3. RFC from rat popliteal lymph nodes. Wet preparations. (A) Small rosette 6 days after inoculation, × 700. (B) Large rosette 6 days after inoculation, × 700. (C) Small rosette 6 days after inoculation, × 315. (D) Large rosette 6 days after inoculation, × 280. (E) RFC exhibiting polar adherence of SRBC, 4 days after inoculation; arrow indicates RFC, × 280. (F) RFC exhibiting polar adherence of SRBC, 4 days after inoculation; note the numerous layers of adherent SRBC attached to the RFC; arrow indicates RFC, × 280.

pressed down firmly pushing the layers of agglutinated SRBC to the sides (Figs 3C and D). These latter photographs emphasize the difference amongst RFC as to the number of adherent SRBC.

RFC with large agglutinates of SRBC were not seen in preparations from control rats. The incidence of such cells in preparations from inoculated rats increased from day 3 to reach a peak between days 5 and 6, followed by a slow decline with a few still being observed 10 days after inoculation.

Although SRBC were usually adherent to the entire cell surface, some RFC exhibited a polar adherence of SRBC as shown in Figs 3(E and F). The latter photograph further illustrates how RFC exhibiting polar adherence can *also* form large agglutinates of the adherent SRBC.

TABLE 1

AVERAGE DIFFERENTIAL RFC COUNT FROM CONTROL AND INOCULATED RATS; RESULTS EXPRESSED AS PERCENTAGE OF TOTAL RFC; THE VALUE FOR DAY 0 WAS DETERMINED FROM TWELVE CONTROL RATS AND FOR EACH SUBSEQUENT DAY FROM SIX INOCULATED RATS

	Days after injection of SRBC										
	0	1	2	3	4	5	6	7	8	9	10
Macrophages	26	12	8	7	6	10	9	15	10	14	16
Haemocytoblasts	0	42	69	64	52	43	32	27	29	23	17
Plasmacytes	0	0	2	6	17	21	35	32	24	21	12
Lymphocytes	74	46	21	23	25	26	24	26	37	42	55

CLASSIFICATION OF RFC BASED ON CELLULAR MORPHOLOGY

On examination of the stained preparations five or more adherent SRBC were again taken as the criterion on which cells were identified as RFC. Drying caused some rosettes to become slightly separated from the central immunocyte, but the pattern of agglutinated SRBC made such an RFC easily identifiable against a background of negative lymph node cells and unagglutinated SRBC. A classification of RFC on morphological grounds was attempted taking into account the following characteristics: cell size, cytoplasmic outline, basophilia, cytoplasmic/nuclear ratio and position, shape and density of the nucleus. The average diameter of a SRBC was measured as 4·8 μ and this value was used to calculate the size of RFC. Based on these criteria RFC were classified as macrophages, haemocytoblasts, plasmacytes and lymphocytes. An accurate classification on morphology alone proved difficult due to variations amongst closely related cell types. However, differentiating RFC was of particular importance in attempts to establish intermediate cell types between two groups. The differential RFC count in both control and inoculated rats is shown in Table 1. In the control lymph nodes only macrophages and lymphocytes were found to form rosettes. In contrast during the 10-day post-inoculation period there was a marked heterogeneity in the morphology of RFC.

1. *Macrophages*

Macrophages formed an average 26 per cent of RFC from the popliteal lymph nodes of uninoculated rats. After inoculation the percentage of rosette-forming (RF) macrophages dropped but later increased from the seventh day to form 16 per cent on day 10.

However, due to the large increase in the number of RFC after inoculation, there was an increase in the *total* number of RF macrophages, e.g. on day 5 there were 1·43 RF macrophages/1000 lymph node cells as compared to 0·18/1000 in uninoculated rats, representing an eight-fold increase. There seemed to be no discernible difference between RF macrophages from control rats and those from inoculated rats and after 2 hours incubation many of these cells were swollen with ingested SRBC (Fig. 4A). A small number of RFC which were recorded as macrophages were found to have little if any ingested material (Fig. 4B). It is possible that these latter cells were not macrophages, but represented another cell type such as reticular cells. It was also noted that many macrophages with ingested SRBC did not form rosettes.

2. *Haemocytoblasts*

No RF haemocytoblasts were seen in the stained films from control rats. 24 hours after inoculation 42 per cent of RFC were identified as haemocytoblasts, this count rising to 69 per cent after 48 hours; after 3 days haemocytoblasts constituted 64 per cent of all RFC, after 4 days 52 per cent and after 5 days 43 per cent. The percentage of haemocytoblasts continued to decrease, 17 per cent being found 10 days after inoculation. These figures must be taken into account with the increase in total RFC/1000 lymph node cells (Fig. 2); for example, after 2 days there were 0·86 RF haemocytoblasts/1000 cells, forming 69 per cent of the total RFC population, and after 5 days there were 6·15/1000, though forming only 43 per cent of total RFC.

Almost all RF haemocytoblasts found during the first 48 hours were large primitive cells, examples of which are shown in Figs 4(C–F). These cells had an indistinct outline and a diameter of 15–25 μ, abundant and basophilic cytoplasm and a large open nucleus often irregular in shape. There was a limit as to the number of SRBC adherent to these early haemocytoblasts although the SRBC that were attached were strongly adherent (Fig. 4C). It was observed that during the first 48 hours there were a considerable number of negative haemocytoblasts with a similar morphology to those RFC described above.

Three days after inoculation the majority of RF haemocytoblasts were more mature cells with a diameter of 15–28 μ, a firm and compact cellular outline, a highly basophilic cytoplasm and a nucleus more regular in shape and usually eccentric. Examples of this cell type are shown in Figs 5(A–F). These latter cells occurred in increasing numbers 48 hours after inoculation reaching a peak at 3 days, then declining rapidly after 4 days although a small number were consistently observed up to the tenth day.

Subsequent to incubation these mature haemocytoblasts had a greater number of adherent SRBC as compared to the more primitive haemocytoblasts found earlier. Indeed on days 3 and 4 a proportion of the mature haemocytoblasts were surrounded by numerous layers of agglutinated SRBC (Fig. 5E) which would seem to indicate an increasing involvement in the immune response.

Small haemocytoblasts first appeared 48 hours after inoculation and then increased in numbers to reach a peak at 5–6 days and from the 5th until the 10th day this cell type formed the majority of RF haemocytoblasts. Small haemocytoblasts were compact and regular shaped cells and had a diameter of 10–15 μ, a variable amount of highly basophilic cytoplasm with the basophilia being particularly noticeable at the edges of the cell and a large eccentric nucleus. Examples of this cell type are shown in Figs 6(A–F). There were many intermediate cell types classed on morphological grounds as small haemocytoblasts, some closely resembling large lymphocytes and others resembling immature

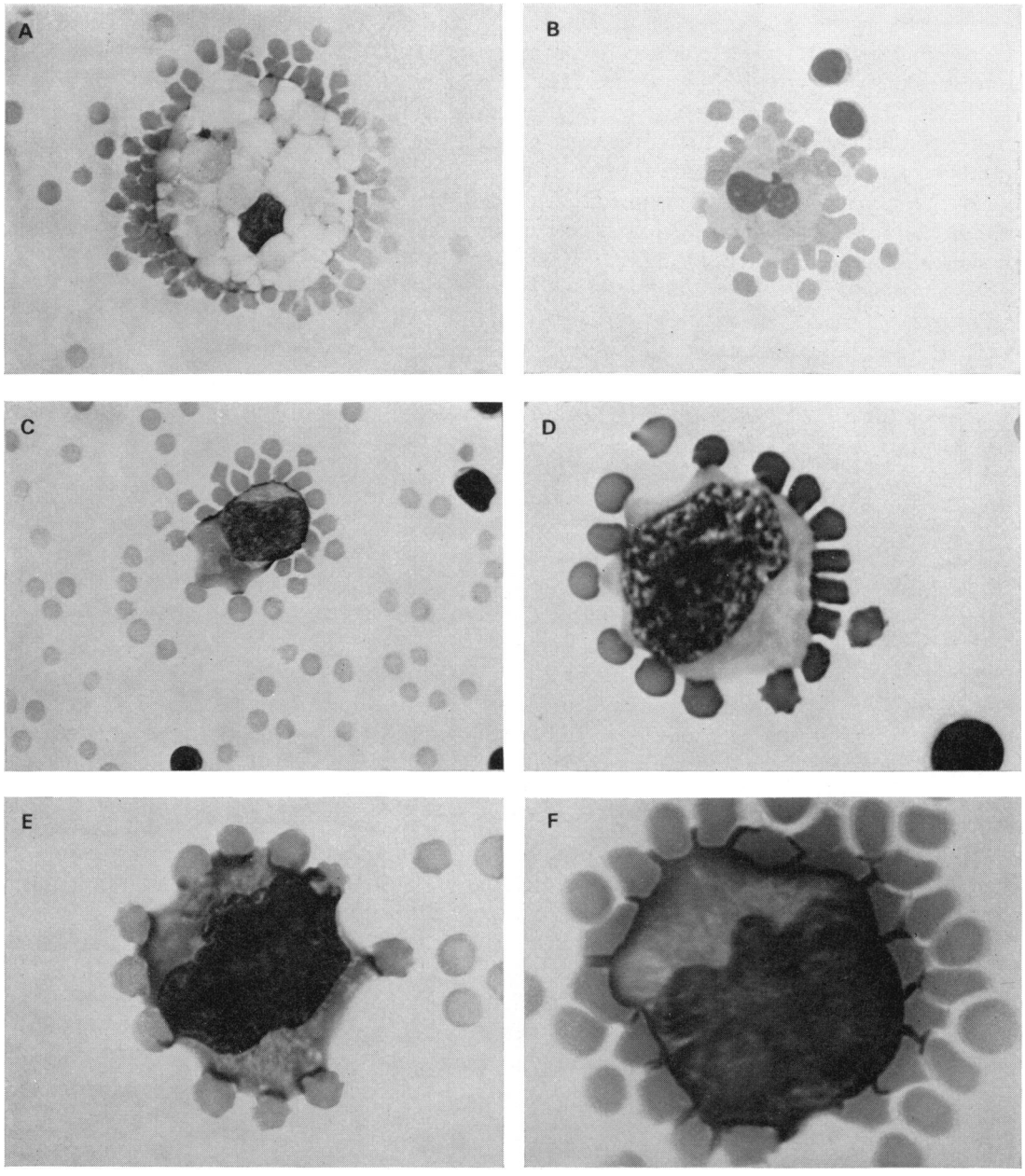

FIG. 4. RFC from rat popliteal lymph nodes. (A) Macrophage from uninoculated rat showing ingestion of SRBC, × 630. (B) Macrophage from uninoculated rat, × 900. (C) Primitive large haemocytoblast 1 day after inoculation, × 630. (D) Primitive large haemocytoblast 3 days after inoculation, × 1120. (E) Primitive large haemocytoblast 2 days after inoculation, × 1120. (F) Primitive large haemocytoblast 2 days after inoculation, × 1260.

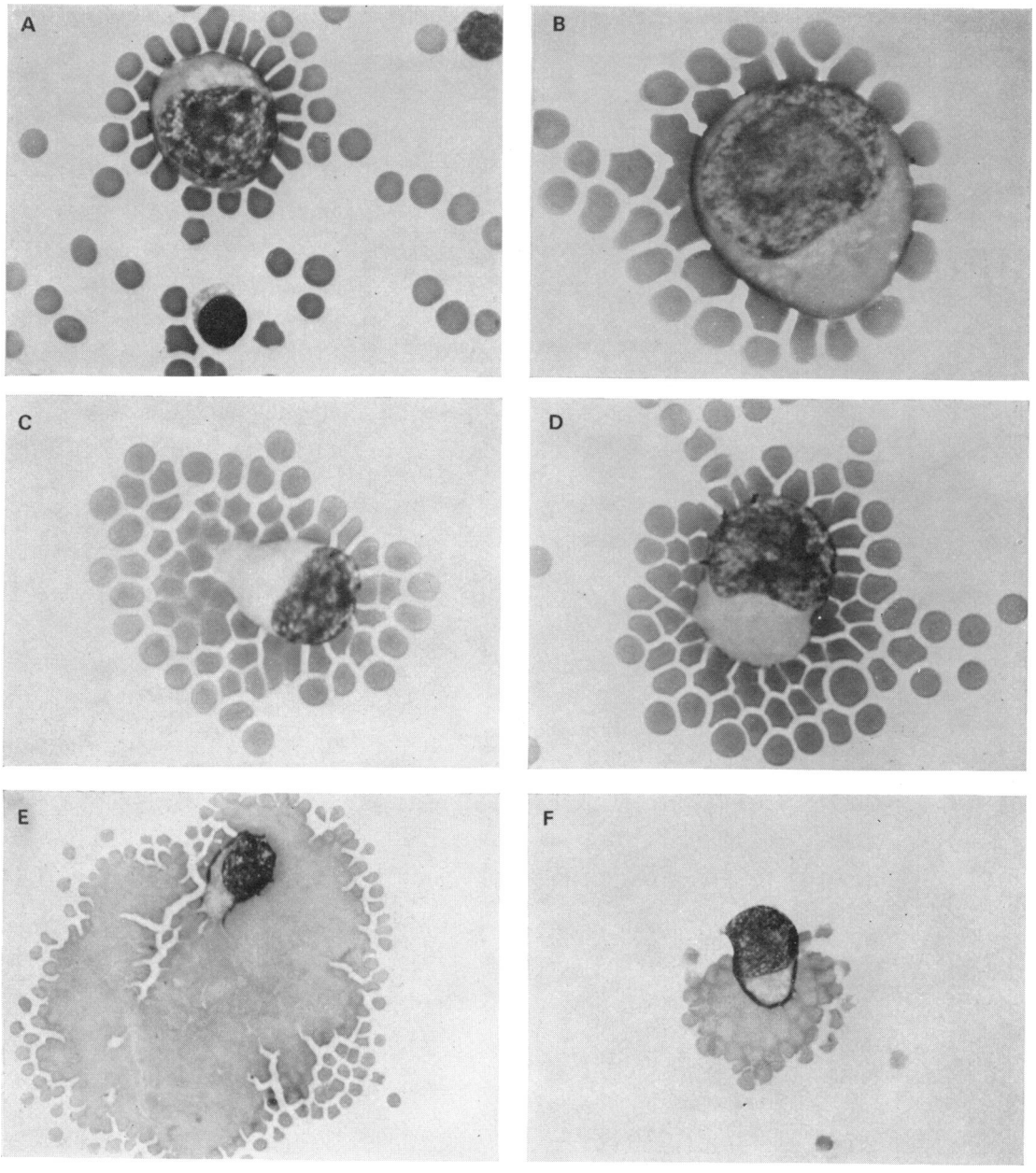

FIG. 5. RFC from rat popliteal lymph nodes. (A) Large haemocytoblast 4 days after inoculation, ×980. (B) Large haemocytoblast 3 days after inoculation, ×1260. (C) Large haemocytoblast 5 days after inoculation, ×980. (D) Large haemocytoblast 4 days after inoculation, ×980. (E) Large haemocytoblast 4 days after inoculation, ×365. (F) Large haemocytoblast 3 days after inoculation; note the polar adherence of SRBC, ×500.

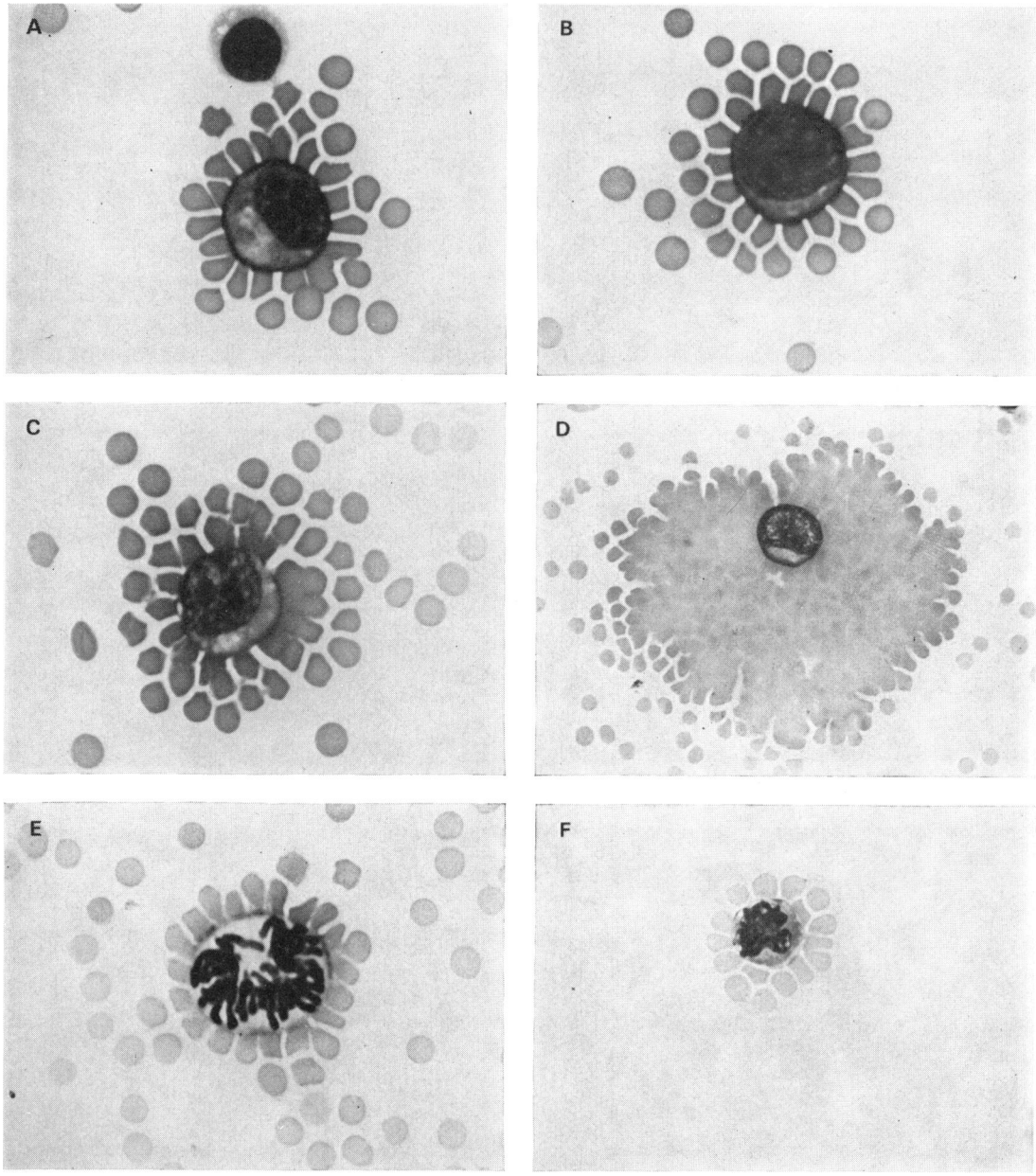

FIG. 6. RFC from rat popliteal lymph nodes. (A) Small haemocytoblast 5 days after inoculation, × 1050. (B) Small haemocytoblast 7 days after inoculation, × 1050. (C) Small haemocytoblast 5 days after inoculation, × 1050. (D) Small haemocytoblast 5 days after inoculation, × 500. (E) Small haemocytoblast in mitosis 3 days after inoculation, × 1050. (F) Small haemocytoblast in mitosis 3 days after inoculation, × 700.

plasmacytes. Small haemocytoblasts normally had one to three layers of adherent SRBC although cells with more than five layers were occasionally observed (Fig. 6D).

3. *Plasmacyte series cells*

No RF plasmacytes were observed in uninoculated control rats. On day 2 and day 3 2 per cent and 6 per cent respectively of all RFC were classified as plasmacytes. After 4 days this figure rose rapidly and peak numbers of RF plasmacytes were found between days 5 and 7 followed by a gradual reduction to 12 per cent on day 10 (Table 1).

Both mature and immature plasmacytes were compact cells with a diameter of 10–15 μ, abundant basophilic cytoplasm and an eccentric nucleus. However, in immature forms (Figs 7A–C) the nucleus was large and often irregular in shape so that the distinction between these cells and some small haemocytoblasts was often imprecise (cf. Figs 6A and 7A). Mature plasmacytes because of their smaller and denser nucleus were more easily identified (Figs 7D–F).

Both immature and mature RF plasmacytes were observed from the 3rd to the 10th day after inoculation, the immature forms being predominant from the 3rd to the 5th day and the mature plasmacytes from the 7th to the 10th day. Immature plasmacytes were usually surrounded by several layers of adherent SRBC (Figs 7A–C). Mature plasmacytes were inconsistent in this respect, some were surrounded by many layers of agglutinated SRBC (Fig. 7F), but more often there were only one to three layers (Fig. 7D).

4. *Lymphocyte series cells*

In the lymph nodes from control rats lymphocytes formed an average 74 per cent of all RFC. 24 hours after inoculation this value had dropped to 46 per cent and to 21 per cent after 48 hours. Lymphocytes then continued to form approximately 25 per cent of all RFC until the 8th day when there was an increase to 37 per cent; this increase was extended to 55 per cent on the 10th and final day. However, although lymphocytes formed the majority of RFC on the 10th day the *actual* number of RF lymphocytes was 3·36/1000 lymph node cells as compared to the 5th day when 26 per cent of RFC were lymphocytes, a value of 3·72 RF lymphocytes/1000 cells. Both small and large lymphocytes were found to form rosettes. They had a diameter of 5–10 μ, a limited amount of cytoplasm and a dense nucleus (Figs 8A–D). There seemed no discernible difference between RF lymphocytes from control rats and those from inoculated rats, although it was possible that many RF small haemocytoblasts could be cells of the lymphocyte series undergoing a process of activation or transformation. Almost all RF lymphocytes had no more than one or two layers of adherent SRBC (Figs 8A–D), although an occasional lymphocyte had more than two layers.

5. *Mitosis*

Mitotic figures were recognized in small numbers 24 hours after inoculation and then with increasing frequency to reach a peak at approximately 3 days. No RFC in mitosis was observed during the first 48 hours, but after 3 days a proportion of RFC were found to be in mitosis (Figs 6E and F). Morphologically these RFC were similar to cells classified as small haemocytoblasts.

6. *Polar adherence of SRBC*

As mentioned above, many RFC observed in wet preparations exhibited polar adherence

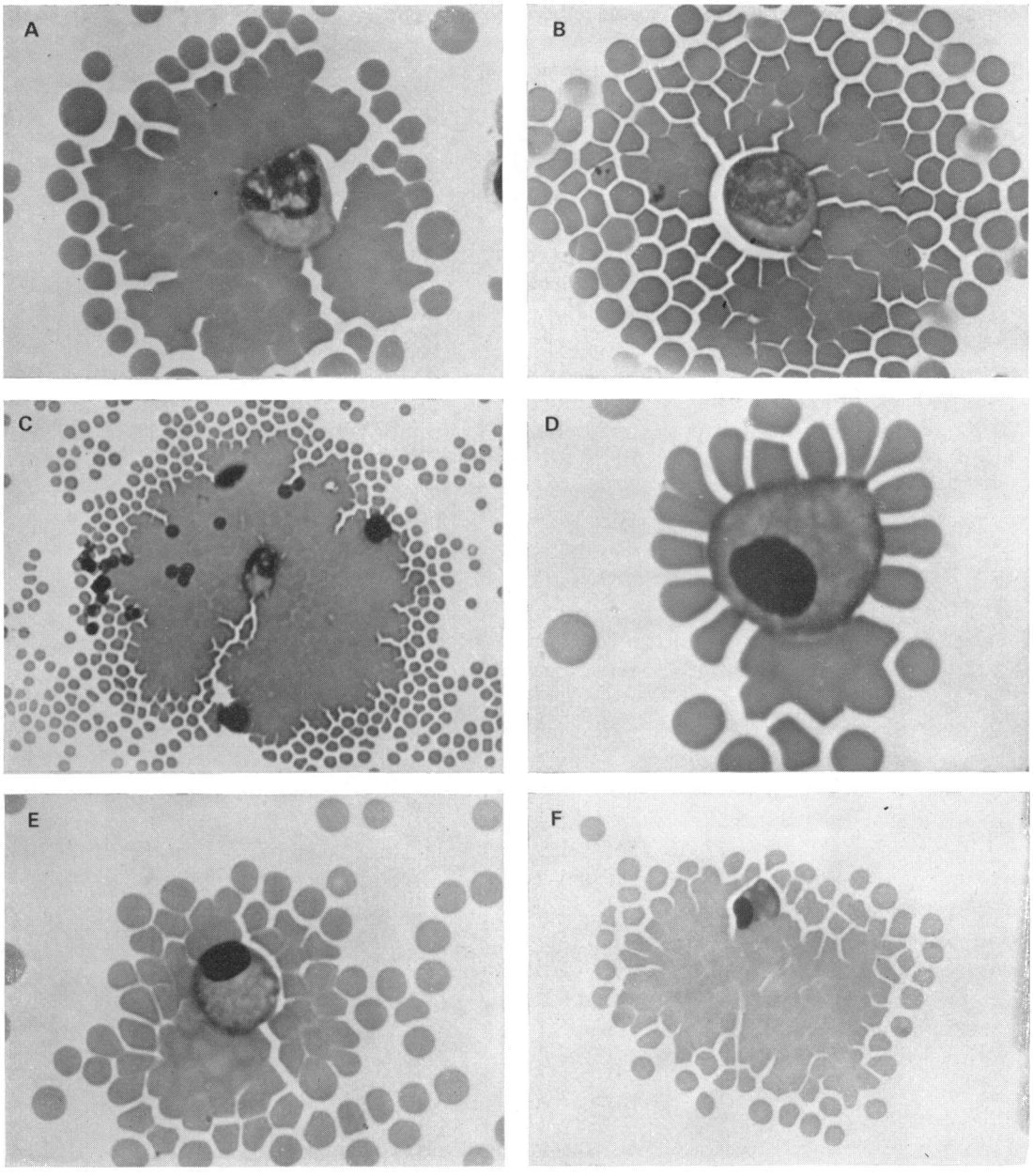

FIG. 7. RFC from rat popliteal lymph nodes. (A) Immature plasmacyte 5 days after inoculation, ×980. (B) Immature plasmacyte 7 days after inoculation, ×980. (C) Immature plasmacyte 7 days after inoculation, ×315. (D) Plasmacyte 8 days after inoculation, ×1400. (E) Plasmacyte 10 days after inoculation, ×980. (F) Plasmacyte 8 days after inoculation, ×630.

of SRBC. On examination of the stained preparations this feature was found predominantly amongst mature haemocytoblasts, both large and small (Fig. 5F). Occasional plasmacytes were also found to exhibit polar adherence of SRBC.

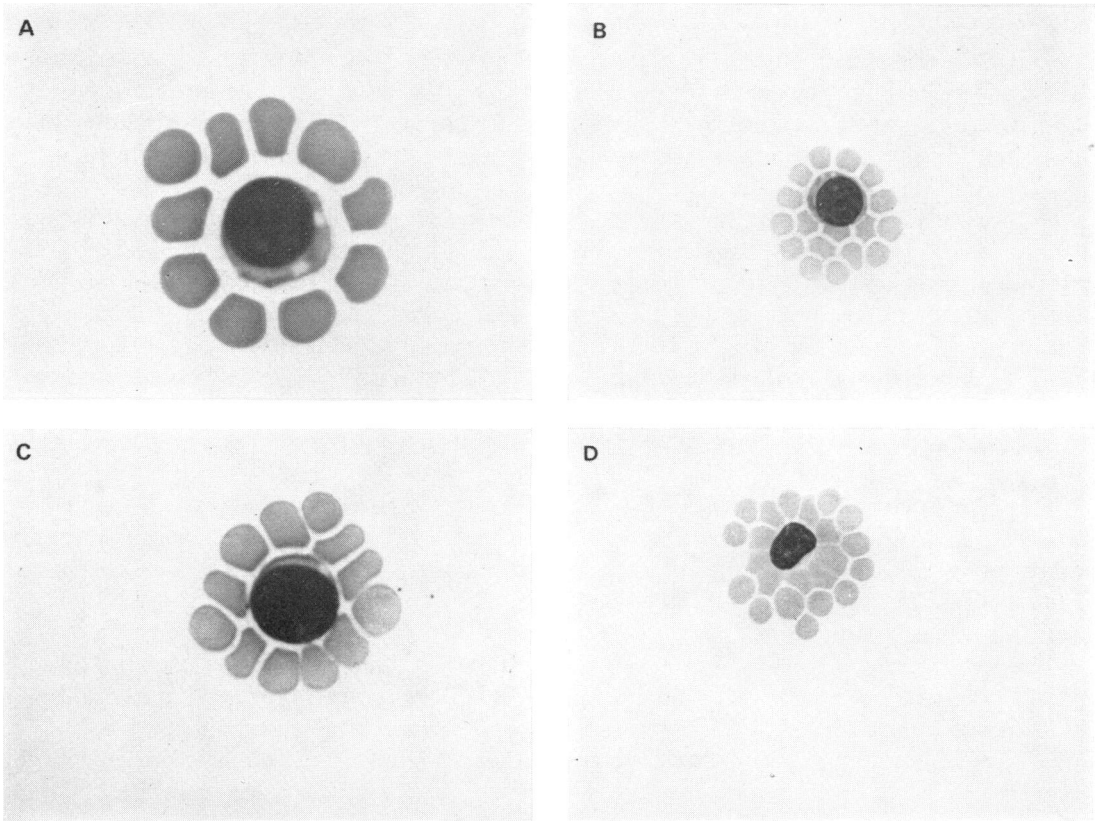

FIG. 8. RFC from rat popliteal lymph nodes. (A) Lymphocyte 6 days after inoculation, ×1400. (B) Lymphocyte from uninoculated rat, ×630. (C) Lymphocyte 8 days after inoculation, ×1400. (D) Lymphocyte from uninoculated rat, ×630.

DISCUSSION

The results of these experiments clearly indicate that incubation of the cell mixture at 37° results in a marked variation in the size of rosettes, i.e. as to the number of layers of agglutinated SRBC around the central RFC. Zaalberg, Van der Meul and Van Twisk (1966) under similar conditions observed this phenomenon and concluded that it was due to specific antibody secreted by RFC beyond the first and subsequent layers of surrounding SRBC. It is difficult to visualize a phenomenon other than the active secretion of haemagglutinin that could result in the formation of two or more layers of SRBC around a RFC. This interpretation is of great importance in attempts to delineate functions to RFC.

In our results lymphocytes and macrophages were the only cell types to form rosettes in uninoculated rats. This result is similar to the findings of McConnell *et al.* (1969) who found that the background RFC count against SRBC in the popliteal lymph nodes of

mice was due to small lymphocytes, and of Storb and Weiser (1968) who demonstrated that lymphocytes were responsible for a similar RFC background in mouse spleen. We found that RF lymphocytes were morphologically indistinguishable from other lymphocytes found in cell suspensions of normal popliteal lymph nodes. There is little evidence as to the function of these background RF lymphocytes although McConnell *et al.* (1969) were of the opinion that they represent a population of cells stimulated by previous contact with cross-reacting antigen. In our opinion there still remains the possiblity that these cells could be immunologically competent cells with appropriate receptor sites for SRBC. An interesting anomaly amongst the control rats used in our experiments was a consistent difference between males and females as to the total number of RFC/1000 lymph node cells; the reason for this discrepancy is not known.

Following immunization there was shown to be an increase in the number of RFC with a peak at days 5–6 followed by a gradual decline still evident 10 days after inoculation. However, the popliteal lymph nodes of immunized rats had a *total* cell population many times that of a normal unstimulated node and although this increase in size was a variable factor, the total number of RFC/lymph node in immunized rats as opposed to the controls was much greater than the figure of RFC/1000 lymph node cells could indicate. Examination of the stained films from inoculated rats revealed a heterogenous population of RFC. Other investigations have previously established the pleomorphism of the cellular immune response (Cunningham, Smith and Mercer, 1966; Harris, Hummeler and Harris, 1966; Hummeler, Harris, Tomassini, Hechtel and Farber, 1966; Haskill, Legge and Shortman, 1969). In this present study we grouped RFC into four categories comprising macrophages, haemocytoblasts, plasmacytes and lymphocytes; whilst recognizing many intermediate forms, sharing similar morphological features, making such a cellular classification a very fluid concept.

The occurrence of RF macrophages is at variance with observations by McConnell *et al.* (1969) who failed to identify them amongst RFC from the popliteal lymph nodes of mice inoculated with SRBC. However, Storb and Weiser (1968) and Biozzi *et al.* (1969) have previously demonstrated macrophage rosettes in both lymph node and spleen cell suspensions from guinea-pigs immunized with either SRBC or dinitrophenyl bovine γ-globulin. The presence of IgG receptor sites on macrophages and their sensitization with anti-erythrocyte antibody is well documented (Lo Buglio, Contran and Jandl, 1967; Huber, Douglas and Fudenberg, 1969). However, in our experimental system any sensitization would have occurred *in vivo*, and the demonstration of RF macrophages in normal lymph nodes and their increase in numbers after inoculation emphasizes the need for further experimental work on the factors involved in rosette formation by such macrophages.

Our results clearly show that after inoculation the initial increase in RFC was due to large primitive haemocytoblasts. This is comparable to the results of Eidinger (1968) and Davies *et al.* (1969) who followed the cellular immune response against SRBC in the popliteal lymph nodes of mice. In our results these primitive RF haemocytoblasts were replaced over the following 2–3 days by a more mature cell 15–28 μ in size, basophilic and with a large eccentric nucleus. It was shown that the early primitive haemocytoblasts had a limited number of adherent SRBC indicative of only a limited capacity for haemagglutinin production, but with the development of more mature forms haemagglutinin production is increased, until 3–4 days after inoculation many mature haemocytoblasts were surrounded by several layers of agglutinated SRBC. Cunningham *et al.* (1966) have

previously demonstrated RF haemocytoblasts from both the lymph nodes and efferent lymph of sheep and Leduc, Avarameas and Bouteille (1968) using electron microscopy have convincingly shown the presence of antibody in haemocytoblasts.

The appearance and differentiation of large haemocytoblasts strongly resembles a sequence of cellular events described by Eidinger (1968) in the popliteal lymph nodes of mice after footpad inoculation with SRBC. In this study Eidinger proposed a process of activation of reticular cells into large blast cells actively secreting IgM. Our results are also in agreement with Eidinger in that mitotic figures occurred only in small numbers during the first 48 hours following inoculation, at a time when the number of large haemocytoblasts was rapidly increasing, therefore it is suggested that the large haemocytoblasts arise without mitosis via a process of cell transformation to develop into active haemagglutinin producing cells by the 3rd or 4th day after inoculation; further experiments are in progress in an attempt to determine what class(es) of immunoglobulin these large haemocytoblasts are secreting.

Within the overall classification of small haemocytoblasts a peak of mitotic activity occurred 3 days after inoculation. Amongst RF small haemocytoblasts many intermediate cell types were recognized with morphological features of either lymphocytes or immature plasmacytes, but an accurate subclassification was not considered possible due to the limitations imposed by light microscopy. Immature RF plasmacytes were shown to be predominant from the 3rd to 5th day and adult RF plasmacytes from the 7th to 10th day. We found no morphological evidence of intermediate RF cell types to indicate a maturation of large haemocytoblasts into plasmacytes. In contrast, within the group of RF small haemocytoblasts, intermediate cell types could suggest a process of lymphocyte activation into small haemocytoblasts, mitotic division and eventual maturation into plasmacytes as previously described by Eidinger (1968). Our results also indicated that although RF small haemocytoblasts did not usually secrete large amounts of haemagglutinin, intermediate forms showed an increasing involvement in haemagglutinin production and from the 5th to the 8th day after inoculation immature plasmacytes were secreting more haemagglutinin as judged by the ICA technique than any other RFC type. In contrast although mature plasmacytes were found to form large rosettes, many were surrounded by only one to three layers of adherent SRBC. One explanation for this occurrence is dependent on the fact that the efficacy of IgM as an agglutinating antibody is many times that of IgG (Del Guerico, Tolone, Ardrade, Biozzi and Binaghi, 1969), therefore after 2 hours incubation RFC secreting IgM will probably form larger rosettes than those secreting IgG; and in the present study immature plasmacytes may be initially producing IgM before 'switching' over to IgG production as mature plasmacytes (Nossal, Szenberg, Ada and Austin, 1964). However, the immature plasmacytes might actually produce more haemagglutinin than adult plasmacytes, the latter perhaps representing an 'end-stage' as far as the secretion of this antibody is concerned.

A further complex question is the precise function of RF lymphocytes. Expressed as a percentage of total RFC the amount of RF lymphocytes dropped to 21 per cent 48 hours after inoculation and remained at approximately 25 per cent until 8 days after which time they increased to form 55 per cent of all RFC 10 days after inoculation. This predominance of RF lymphocytes in the latter part of an immune response has already been demonstrated in mice (McConnell *et al.*, 1969) and in chickens (Duffus and Allan, 1969). The majority of RF lymphocytes were surrounded by only one or two layers of adherent SRBC, indicating that these cells were not involved to any extent in haemagglutinin production. We had no

evidence as to the function of these RF lymphocytes although presumably a proportion were involved in cellular immunity and immunological memory.

We found that many RF haemocytoblasts and occasional RF plasmacytes exhibited a polar adherence of SRBC. This phenomenon has been previously described in mice by Storb *et al.* (1969) who in contrast to our findings found that polar adherence occurred predominantly amongst lymphocytes. Biozzi *et al.* (1969) found that when RFC from guinea-pigs were stained with fluorescent anti-γ-globulin only a portion of the RFC became fluorescent. Therefore some RFC exhibiting polar adherence might be secreting haemagglutinin at a particular site on the cell surface, although Storb *et al.* (1969) thought that the highly motile cell membrane of some RFC might produce a selective adherence of SRBC.

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