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The Immune Response to Sheep Erythrocytes in the Mouse

II. A STUDY OF THE CYTOLOGICAL EVENTS IN THE DRAINING LYMPH NODE UTILIZING CELLULAR IMPRINTS

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Summary. The cytological events of the primary and secondary immune response in the popliteal lymph node of Swiss White mice were studied following administration of sheep erythrocytes into the hind footpad. Four morphological features of cellular activity of immunologically competent cells—basophilia, synthesis of RNA, mitotic activity and distinctive cellular morphology—were analysed, and correlated with previous studies of 19S and 7S antibody forming cellular activity employing plaque assays performed on the residual lymphoid tissue remaining after production of node imprints.

The findings support the view that 19S and 7S antibody forming cells in the primary immune response are derived from two populations of cellular precursors. It is suggested that the lymphoid cell producing 19S immunoglobulin arises by transformation from the reticular cell following activation by antigen, while the 7S antibody forming cell arises from the small lymphocyte following some degree of initial transformation and subsequent cellular proliferation. The possibility that the 7S antibody forming cells had passed through a transient period of biosynthesis of 19S antibody was suggested in the present studies. Finally, evidence was provided for the presence of two morphological types of plasma cells, which, by virtue of their appearance at different stages of the primary immune response, could represent cells producing different immunoglobulins at varying rates of protein biosynthesis.

INTRODUCTION

Recent investigations of the cytological events in the immune response implicate two populations of cells in the production of 19S and 7S immunoglobulins (Sahier and Schwartz, 1966; Moore, Mumaw and Schoenberg, 1965). A feature of these investigations has been the use of immunosuppressive methods for the inhibition of the 7S immune response, in order to isolate and analyse the morphological characters of the immune response manifested by a multiplicity of cell types and interactions.

Previous work in this laboratory, utilizing the technique of localized haemolysis in gel, indicated that the peak of 19S cellular response in the draining popliteal lymph node occurred on day 4, following primary intradermal (footpad) injection of 2×10^8 sheep erythrocytes, while the initial 7S peak occurred on day 6 (Eidinger and Pross, 1967). The determination of the time of peak responses permitted the analysis of cellular changes in lymphoid tissues containing a maximally stimulated population of cells producing 19S and 7S antibody. Similar studies of the cellular responses prior to and subsequent to these

peak responses provided experimental data upon which an analysis of the origin and fate of these antibody forming cells could be based.

In the present work, the morphological changes in the draining lymph node were determined by examining cellular imprints of the popliteal lymph node after primary and secondary immunization via the hind footpad. Cellular morphology was preserved with great clarity in these preparations. Moreover, the residual lymphoid tissue which remained after production of imprints provided the cell suspensions upon which the numbers of 19S and 7S antibody forming cells were determined by techniques of localized haemolysis in gel (Jerne, Nordin and Henry, 1963; Dresser and Wortis, 1965). This would not have been possible had histological fixatives been used for the preparation of cytological specimens. The analysis of the immune response represented by the correlation of numbers of plaque-forming cells contained in various lymphoid tissues with the amount and type of circulating immunoglobulin was the subject of a previous publication (Eidinger and Pross, 1967). In the present paper, the cytological events in the draining lymph node were analysed and correlated with numbers of plaque-forming cells contained in the same lymphoid tissue.

MATERIALS AND METHODS

Animals

Swiss white mice were utilized in these experiments. Young, adult female animals 5–6 weeks of age were injected with 2×10^8 sheep erythrocytes into the right hind footpad.

The cellular responses in the draining popliteal lymph node of groups of five to ten animals were studied daily for 14 days, and intermittently for 240 days following a single injection into the hind footpad. In addition, groups of animals were re-injected at intervals in the identical site, following which the cellular changes associated with the secondary immune response in the popliteal lymph node were analysed.

Cytology

Imprints of the popliteal lymph node were made by touching the cut surface of the tissue gently to a scrupulously clean glass slide. The slide was then quickly air-dried, fixed in absolute methanol for 3 hours, and then either stored or stained immediately with May–Grünwald–Giemsa stain.

Imprints were also stained with acridine orange, 1 part per 50 parts of a stock dye (purchased from Hartman-Leddon Company, Philadelphia) diluted in distilled water. The histochemical specificity of acridine orange for staining of RNA and DNA was checked by prior treatment of imprints with highly purified preparations of RNAase and DNAase. The orange-red coloration indicative of RNA (Fig. 1) was abolished by pre-treatment with RNAase (Fig. 2), while the green coloration indicative of the presence of DNA (Fig. 1) was abolished by incubation of the imprints with DNAase prior to staining (Fig. 3). Although acridine orange staining is a frequent tool of virologists for the analysis of viral infected cells (Pollard and Starr, 1962), this technique has not to my knowledge been utilized previously in the study of immunologically competent cells. Its reproducibility, and the clarity with which cytological details are revealed by its use, make it a useful adjunct in the study of morphology of immunogenic cells.

Microscopy

A Reichert Zetopan Research Microscope provided with a quartz iodine light source

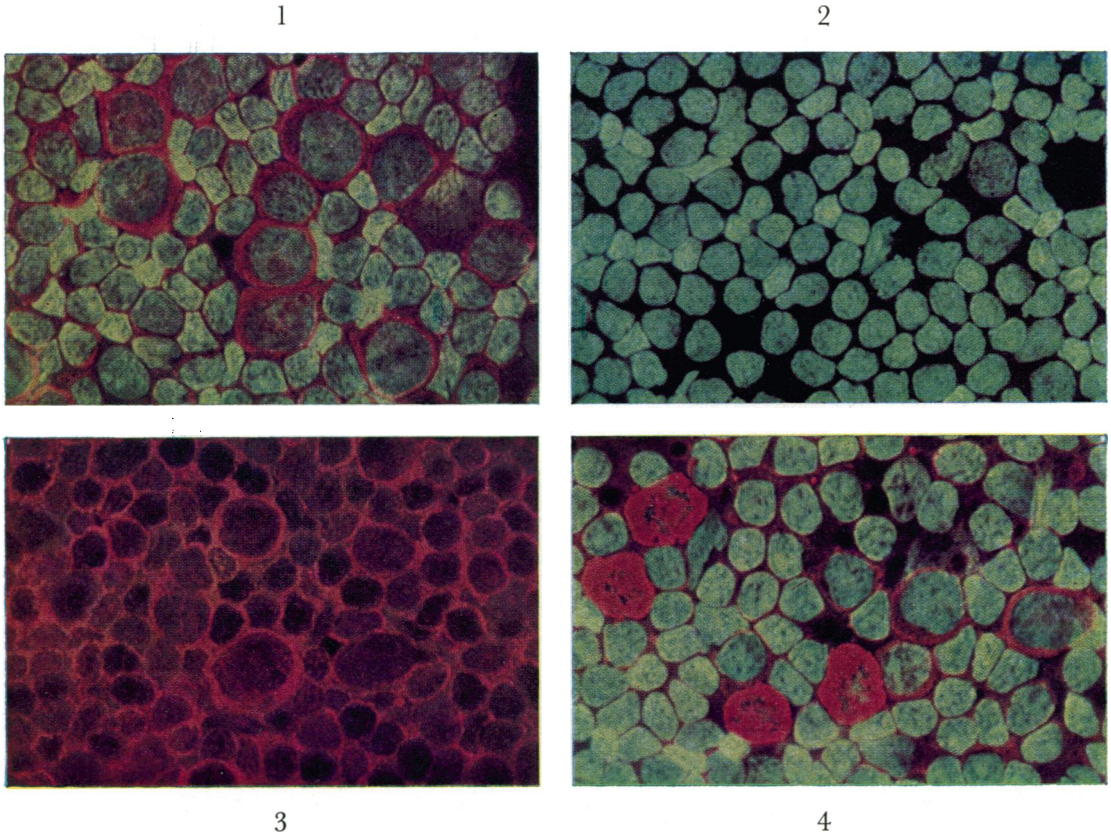

FIG. 1. Cellular imprint of popliteal lymph node on day 2 of the primary response. The green and orange-red coloration is readily visible denoting DNA and RNA respectively contained in activated lymphocytes. × 1250.

FIG. 2. Cellular imprint stained with acridine orange after treatment with RNAase. The red cytoplasmic staining has disappeared. × 1250.

FIG. 3. Cellular imprint stained with acridine orange after treatment with DNAase. The green nuclear staining has disappeared. × 1250.

FIG. 4. Cellular imprint of unimmunized control animal. Four mast cells staining a bright red are visible, as well as a small number of medium and large lymphocytes. Such infiltrates occurred in 5 per cent of normal animals (see text). × 1250.

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was used in the present work. Black and white photographs were taken on Adox KB 14 film, with blue and green diffusion filters to heighten contrast. Colour photographs of acridine orange preparations were taken on Kodachrome X daylight type film.

Cell measurements were made utilizing a Bausch and Lomb eyepiece micrometer and a Reichert stage micrometer.

RESULTS

THE RESPONSE IN UNIMMUNIZED CONTROL MICE

The cytology of normal popliteal lymph node imprints was characterized by a nearly homogenous population of small lymphocytes measuring 8–11 μ (Fig. 4). The majority of these cells were of a typical appearance, while a small minority exhibited some features of degenerative change in that the cells were smaller (8–9 μ), the nuclei were somewhat shrunken and contained numerous dense chromatin particles. Very small numbers of medium and large lymphocytes and inactive reticular cells were observed. In addition, a number of polymorphonuclear leucocytes, eosinophils, and mast cells were seen in some of the lymph node imprints (Fig. 4). In about 5 per cent of the animals examined, the numbers of medium and large lymphoid cells were substantial (3–5 per high power field). These findings were attributed either to lymphocytopoiesis or to intrinsic immunological reactivity. Small numbers of typical plasma cells were observed in only two normal imprints of about 100 examined.

THE RESPONSE IN ANIMALS AFTER PRIMARY IMMUNIZATION WITH SHEEP ERYTHROCYTES

The earliest detectable change in the cytology of the draining lymph node was the sequence of cytological events which was interpreted as indicative of the process of activation of reticular cells (Fig. 5). These changes were noted initially in the lymph node sampled at 24 hours following primary immunization, and were readily demonstrable after 48 hours. The process of activation of reticular cells was characterized by an increase in nuclear size, associated with the appearance of nucleoli which stained red in the acridine orange preparations indicating the presence of RNA. The cytoplasm, which in the resting reticular cell was foamy, virtually unstained, and possessing no discernible cytoplasmic membrane, began to exhibit basophilia which increased in intensity with time, while the cytoplasmic membrane became more sharply delineated. The pattern of activation of the reticular cells became complete with the appearance at 48–96 hours after immunization, of large lymphoid cells around 23–27 μ in size, possessing nuclei 20–25 μ in size. The cytoplasm of these cells as revealed by May–Grünwald–Giemsa was intensely basophilic, and with acridine orange stained a bright red, denoting RNA. A large number of these cells contained one or more nucleoli which also stained red in acridine orange preparations.

Two days after primary immunization, a pattern of cytological events interpreted as representative of the transformation of small lymphocytes to large blast cells was ascertained. Fig. 6 demonstrates the sequence of changes in size and morphology in this population of cells, which was attributed to cellular activation. The small lymphocytes measuring 9–11 μ gradually increased in size, and were associated with increasing basophilia and RNA production as manifested by staining reactivity with acridine orange. Nucleoli appeared and the clumping of nuclear chromatin was less pronounced. Cells

continued to enlarge until they had attained the size of large blast cells measuring 24 μ, similar in size and nuclear–cytoplasmic ratio to those cells produced by reticular cell activation. The cells could not readily be differentiated from the latter except, possibly, by a slightly enhanced basophilia. Fig. 7 also illustrates that mitotic activity was present throughout this line of cells, since cells of each size were observed in mitosis. While mitotic activity of medium and large cells in excess of 17 mμ in magnitude predominated, mitotic activity amongst smaller cells measuring 12–14 μ was not rare.

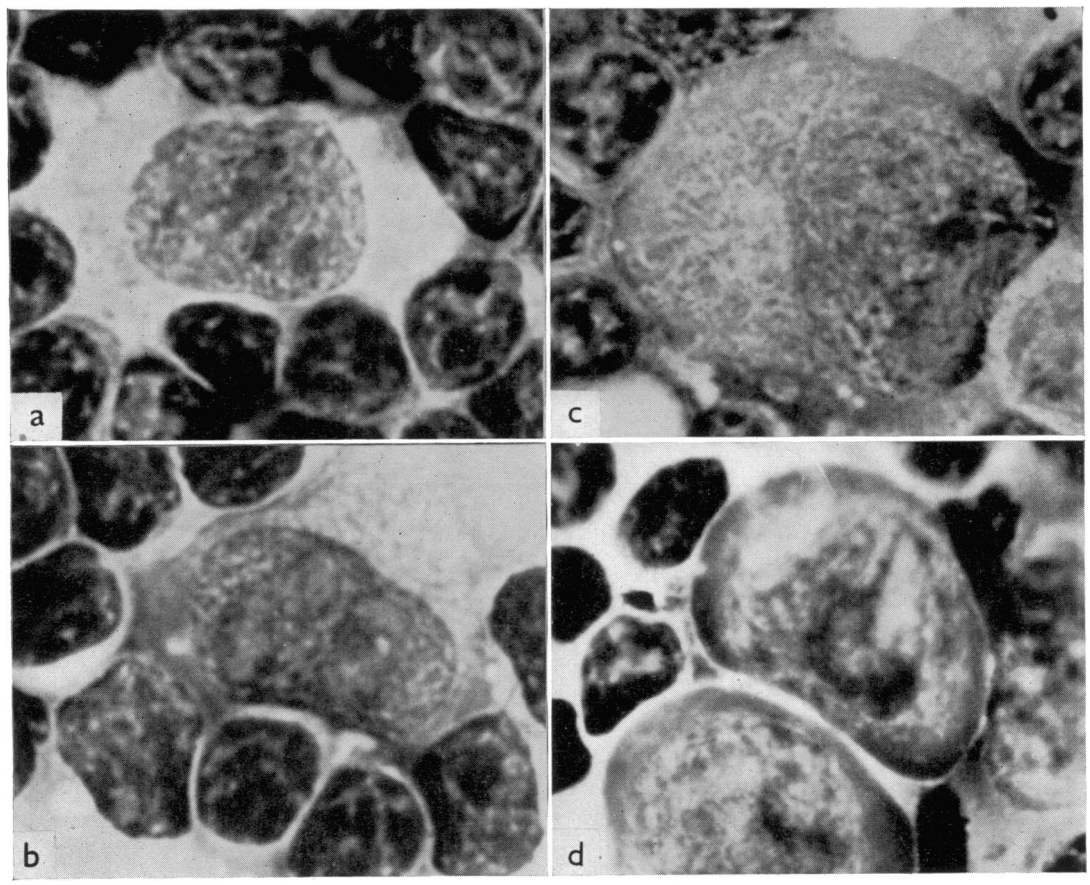

FIG. 5. Composite picture of activation of reticular cells from a resting state (a) to an immunoblast (d) occurring 24–48 hours after primary immunization. ×4000.

The cytological events which characterized transformation of small lymphocytes were noted on the 2nd and 3rd days following primary immunization. Subsequent to day 3, the transformation of small lymphocytes to large lymphoid cells was obscured by dividing cell populations, which generated large numbers of smaller lymphoid cells from large blast cell precursors. It was previously found that the peak of 19S plaque forming activity in the popliteal lymph node cells occurred on day 4 of the primary immune response (Eidinger and Pross, 1967). At this time, a preponderance of large and medium-sized lymphoid cells measuring in excess of 17 μ was noted. The numbers of these cells diminished on the 5th day following primary immunization, concomitant with the appearance of

larger numbers of smaller active lymphoid cells 13–17 μ in size exhibiting intense basophilia and containing RNA. The range of nuclear–cytoplasmic ratios of these cells was 3·25–8·5.

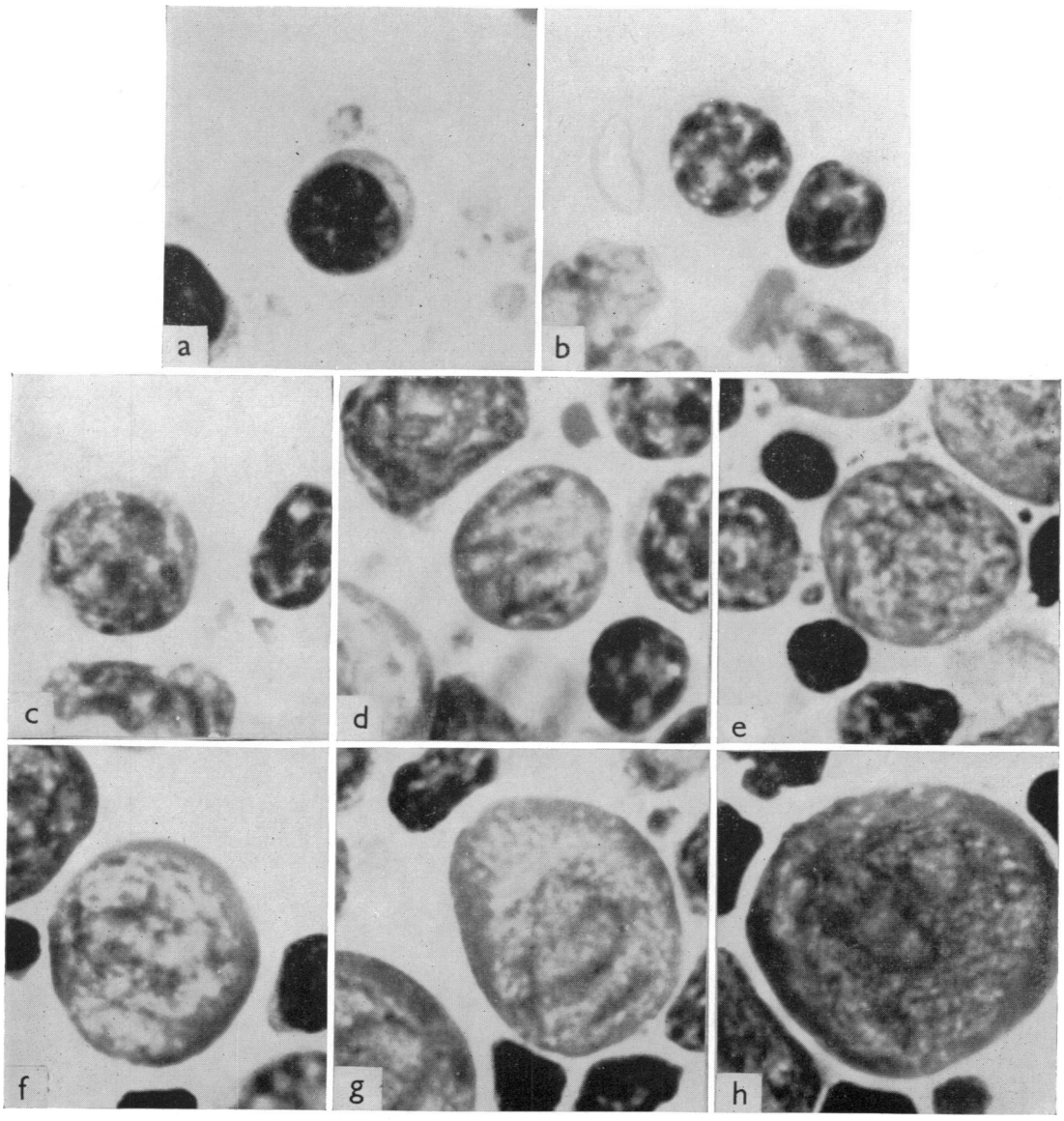

FIG. 6. Composite picture of activation of small lymphocytes from a resting state (a) to an immunoblast (h) occurring 48–72 hours after primary immunization. (e–h) are representative of the morphology of cellular infiltrates associated with 19S antibody synthesis on day 4. ×4000.

The initial peak of 7S antibody synthesis in the draining lymph node cells as revealed by the plaque technique, occurred on the 6th day of the primary immune response. The cellular infiltrates on day 6 were characterized by the presence of an immunogenic cell

exhibiting many of the classical features of plasma cells (Fig. 8a). These cells measured 13–17 μ in size and possessed nuclear–cytoplasmic ratios of 1·5–3·5. On the 7th day large

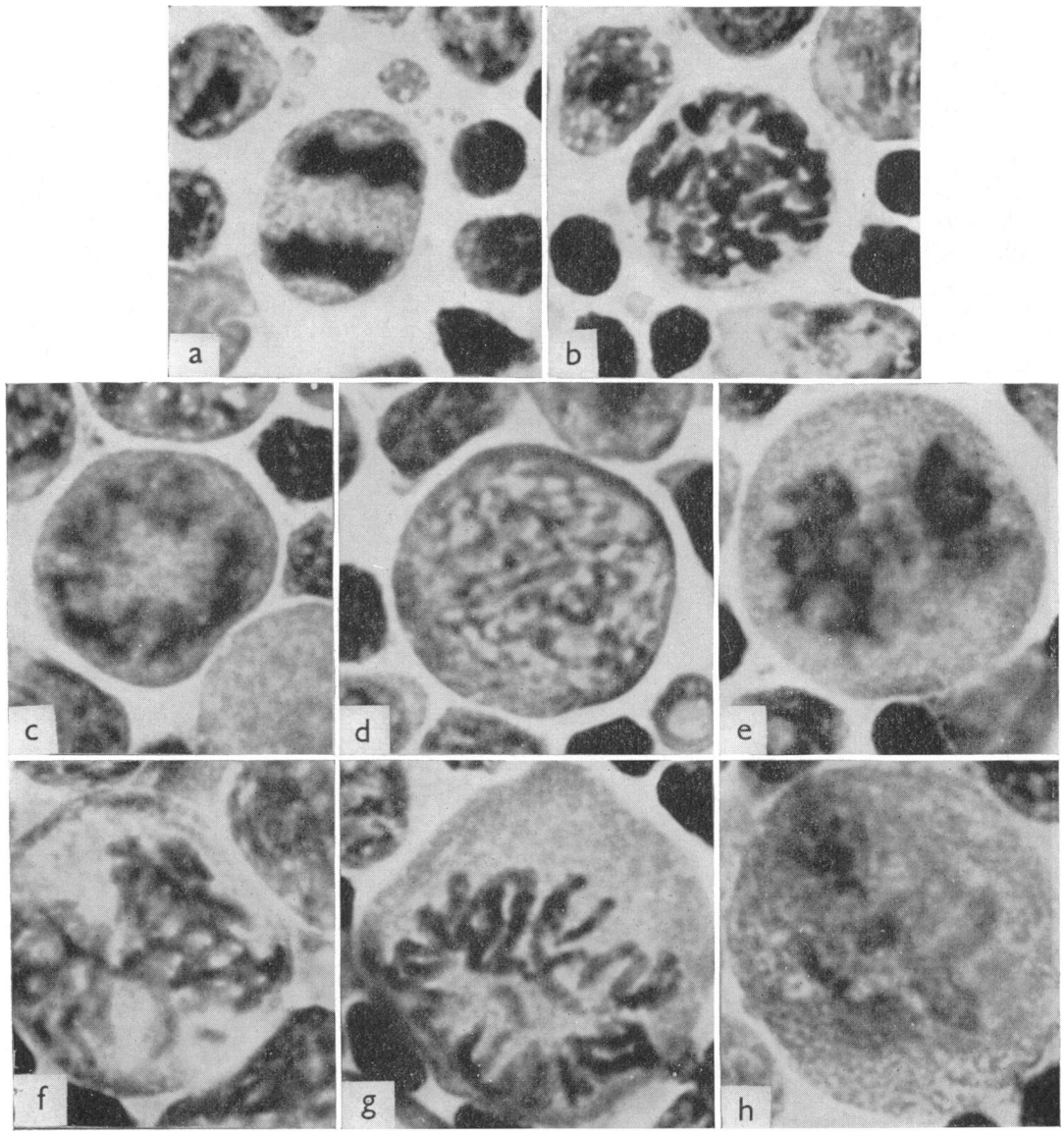

FIG. 7. Examples of mitotic activity of each cell type associated with activation of small lymphocytes. Mitoses in cells exceeding 17 μ in size predominated. ×4000.

numbers of pycnotic, degenerated cells were present, presumably the plasma cells observed on day 6.

The cytological pattern of response subsequent to day 7 was characterized by an admixture of diminishing activities in the two populations of cells, activities which had

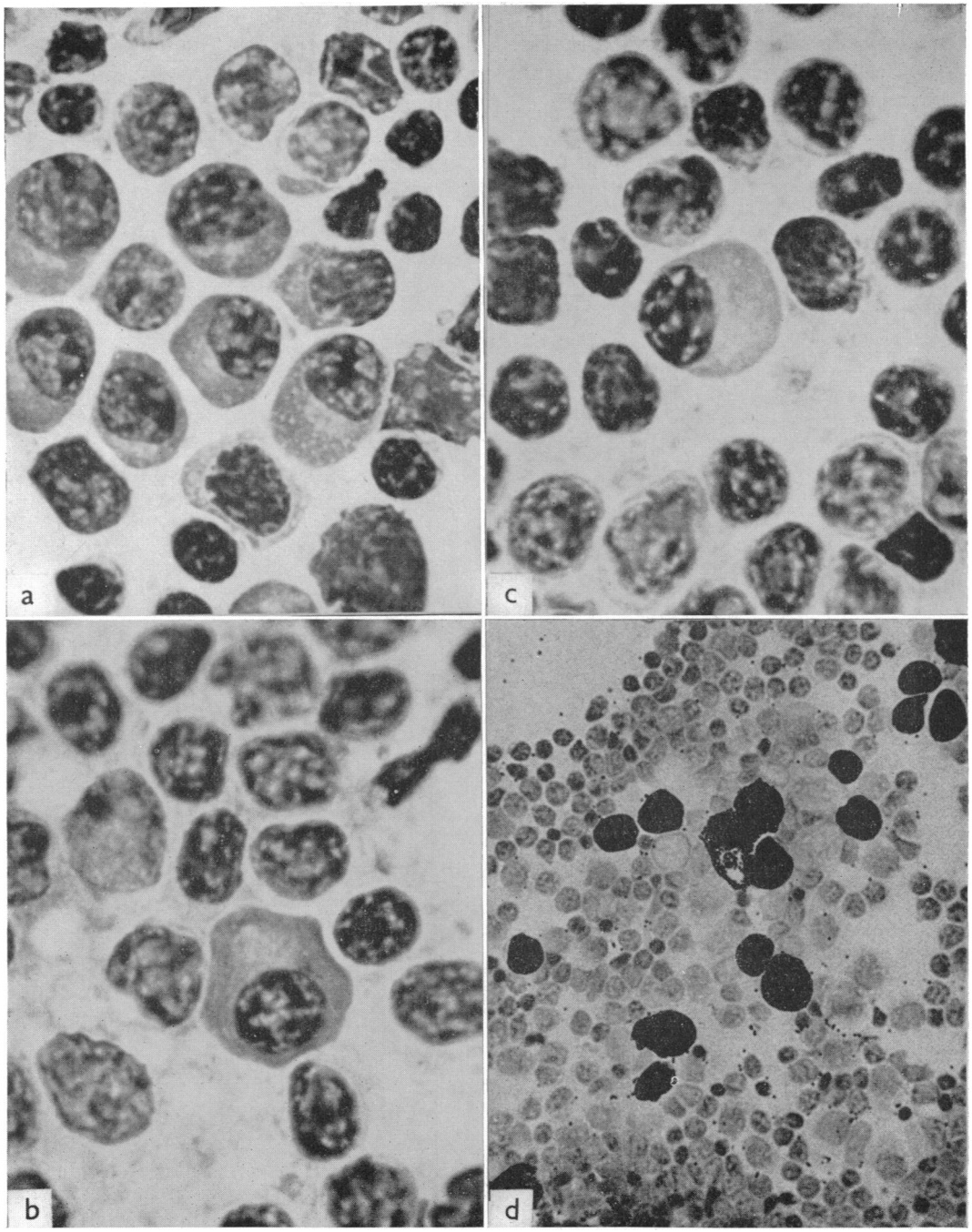

FIG. 8. (a) Cellular infiltrate on day 6 of the primary response. Cells designated as plasma cells are noted in association with initial peak of 7S cellular antibody synthesis. ×2000. (b) and (c) Examples of plasma cells observed during late phases of the primary immune response 80 or more days after primary immunization. Compared with the plasma cells seen on day 6 (a), the major difference lies in the more abundant cytoplasm. ×2000. (d) Cellular infiltrate 160 days after primary immunization demonstrating very large numbers of mast cells. ×200.

been readily differentiated on days 5 and 6 of the primary immune response. The numbers of active lymphoid cells decreased markedly following day 12, and by the 3rd week, the lymph node was nearly normal in morphological appearance.

Throughout the remaining phases of the primary immune response, two further cytological events were observed. It was noted that a small number of plasma cells were present in about half the imprints of the draining popliteal lymph node. These cells differed morphologically from the plasma cells which characterized day 6 of the response

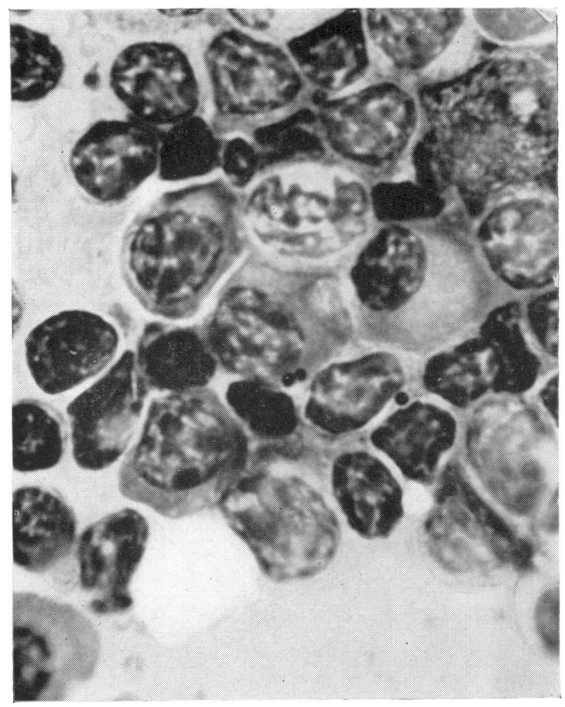

FIG. 9. Cellular infiltrate observed in the popliteal node 5 days after secondary immunization, 140 days after primary injection. One 'late type' and several 'early type' plasma cells are seen. ×2000.

in that they contained a more abundant cytoplasm yielding measurements of nuclear–cytoplasmic ratios of 0·8–1·2 (Fig. 8b and c). The appearance of these cells, which never numbered more than a few per lymph node imprint, was associated with the appearance of a non-IgM, mercaptoethanol sensitive immunoglobulin (Eidinger and Pross, 1967). Moreover, the plaque-forming cellular activity had decreased to barely more than background by the 60th day following primary immunization.

It was also observed on the 60th day and subsequently, that the lymph nodes of a majority of animals contained larger numbers of mast cells than the controls, with as many as fifteen cells per low power field (Fig. 8d), as compared to not more than four cells per field in the non-immunized animal (Fig. 4). Although a similar correlation with the change in circulating immunoglobulin and marked diminution in plaque forming cellular activity could be made, the function of these cells and their relationship to these alterations are unknown.

THE CYTOLOGY OF DRAINING POPLITEAL LYMPH NODES IN SECONDARILY STIMULATED ANIMALS

The cell population of lymph node imprints performed on day 4 or 5 of the secondary response corresponded to the maximum number of 7S plaque forming cells obtained by plaque assay (Eidinger and Pross, 1967). Two morphological types of classical plasma cells were observed on days 4–5; the one, more numerous, corresponding to those observed on day 6 of the primary immune response, and the other, present in smaller numbers of the type which characterized the late phase of the primary immune response (Fig. 9). Many more cells of this latter type, however, were observed in the secondary response than in the late phase of the primary response.

Cellular imprints performed prior to day 4 demonstrated an admixture of multiple cell types which made interpretation of the origin of the plasma cells observed at the time of maximal plaque forming activity difficult. In general, larger numbers of eosinophils, polymorphonuclear leucocytes, and mast cells were observed than in the cellular infiltrates which characterized the early part of the primary immune response.

DISCUSSION

The immune response in living hosts may be subdivided into two distinct biological stages, the first associated with the recognition and processing of antigenic substances, and the second manifested by a specific proliferation of clones of cells engaged in the production of immunoglobulins. These immune manifestations are characterized by four morphological features which represent the hallmark of cytological manifestations of many kinds of cells engaged in active biosynthesis. The first characteristic, which is termed basophilia, has been utilized for many years by morphologists in characterizing the biological activity of many kinds of cells engaged in secretory activity (Ham, 1965). Cytoplasmic basophilia represents the affinity of basic dyes for structures containing RNA and in this respect, it is a representation of the second characteristic of cells engaged in active biosynthesis of protein, namely, the synthesis of RNA. The usefulness of acridine orange in demonstrating this second characteristic of active cells has been demonstrated on frequent occasions by virologists, in their studies of the biosynthesis of viral components in infected cells (Polland and Starr, 1962), and its use in the current investigation exemplifies its potential in the study of immunogenic cells. The third morphological characteristic representative of activation of immunologically competent cells, is the mitotic activity of clones of cells recruited by the administration of antigen. The fourth feature, which is distinctive for the immunological event, is the morphology of the types of immunologically competent cells which are responsible for the biological manifestations of the event.

The four morphological features denoting activation of immunologically competent cells were analysed in the present work. Since 95 per cent of the resting popliteal lymph nodes in young Swiss White mice contained very few cells exhibiting basophilia, very few cells containing an abundance of RNA as demonstrated by acridine orange staining, and only an extremely rare mitotic figure or plasma cell, the performance of the present investigation was greatly simplified. The availability of the technique of localized haemolysis in gel which yielded data on the immunological reactivities of the same populations of cells upon which the morphological studies were performed, was of great value in analysing the cellular events of the immune response.

The initial morphological event demonstrated in the popliteal lymph node following

administration of sheep erythrocytes into the hind footpad was the sequence of cellular events ascribed to activation of reticular cells. The end stage of reticular cell activation was the production of a large blast cell, which may be designated an immunoblast. The morphological evidence would suggest that reticular cells might give rise to immunoblasts without the mediation of a mitotic process.

Several groups of workers have designated the reticular cell as the precursor of the immunoblast (Marshall and White, 1950; Nossal and Mäkelä, 1962; Sainte-Marie and Coons, 1964). It would appear that the reticular cell, at least during the initial phases of immunization, is capable of phagocytosis of antigen (Wellensiek and Coons, 1964), and this might provide the trigger for the process of activation of this cell line. Nossal and Mäkelä (1962) provided evidence, utilizing a radioactive tracer for labelling cellular precursors, that the antibody forming cells in the secondary immune response are derived from a population of dividing reticular cells. No information is available for the origin of antibody forming cells in the primary immune response. Thus, it has not been established beyond doubt whether or not the mitotic process is essential for the activation of reticular cells from which immunoblasts are derived. However, recent evidence utilizing colchicine in experiments *in vitro* suggests that activation of immunogenic cells synthesizing 19S antibody can occur without the mediation of mitosis (Bussand and Luvie, 1967), although the identity of the precursors was not investigated. Others have demonstrated the failure of immunosuppressive agents such as lethal irradiation or 6-mercaptopurine to abolish the 19S primary antibody response (Smith and Robbins, 1965; Sahiar and Schwartz, 1965) utilizing dosage levels that completely suppress the 7S response. These agents act by markedly suppressing mitotic activity, indicating that cell division may not be required for 19S antibody production.

Alternatively, it can be argued that the reticular cells have nothing at all to do with antibody formation in the primary immune response, in that these cells might be producing a population of small lymphocytes. The origin of small lymphocytes from reticular cells has been well documented (Sainte-Marie and Leblond, 1958). However, if one compares the degree of basophilia and intensity of acridine orange reactivity in normal control and immunized animals, the intensity of basophilia and content of RNA is greater in activated reticular cells and large blast cells following immunization than in similar cells in the normal control, in which lymphocytopoiesis is occurring. Moreover, it is the administration of antigen that acts as a triggering mechanism for the alteration in morphology and increase in numbers of these cells. On this basis, it is suggested that the activation of reticular cells is a process not merely responsible for the production of populations of small lymphocytes.

Since activation of reticular cells represents the initial apparent morphological event, and since this process evolves to its maximum level at the time of development of maximal 19S antibody synthesis, the immunoblasts which are derived from reticular cells by activation, and any progeny of these cells which arise subsequently by mitosis are thought to be responsible for 19S antibody formation. The correlation of circulating 19S antibody with the appearance of large pyroninophilic mononuclear cells in the spleen (Sahier and Schwartz, 1966), the demonstration by immunofluorescence of 19S antibody forming non-phagocytic, mononuclear cells in the red pulp of the spleen (Moore *et al.*, 1965), and the correlation of peak 19S plaque forming cellular activity with the appearance of maximum numbers of these cells (Eidinger and Pross, 1967) provide further experimental evidence for this view.

The second process of cellular transformation observed in the present work is in the population of small lymphocytes. The morphological characteristics which were attributed to activation and transformation of small lymphocytes were increase in cell size, increased stainability with acridine orange interpreted as indicating RNA synthesis, and alteration in nuclear morphology characterized by appearance of nucleoli and increase in nuclear size. In many of these characteristics, the activation of these cells mirrors the morphological changes observed with lymphocyte activation *in vitro* utilizing pokeweed mitogen (Chessin, Borjeson, Walsh, Douglas and Cooper, 1966). Similarly, the indications of RNA synthesis in a population of immunogenic cells at a time when mitotic activity was not a striking morphological feature, correlates with the *in vitro* demonstration of biosynthesis of RNA prior to the onset of DNA synthesis in the cells being transformed by pokeweed mitogen (Chessin *et al.*, 1966).

In the present work, transforming cells were observed in moderate numbers prior to the onset of 19S antibody formation demonstrated by plaque assay on day 3 of the primary immune response. In terms of biosynthesis of RNA, this period is analogous to the peak of RNA synthesis of the secondary immune response studied by Cottier, Odartchenko, Keiser, Hess and Stoner (1964). These workers noted maximal incorporation of labelled cytidine into RNA on day 2, some 24 hours prior to the initial peak of thymidine incorporation into DNA.

These experiments suggest that the population of small lymphocytes activated by antigen, transform without the intervention of a mitotic process at least during the initial stages. The nuclear changes associated with transformation, such as formation of nucleoli, decrease in clumping of chromatin and increase in nuclear size are cytological features of cells that have enlarged to a magnitude of 17 μ, after which, morphological assessment of representative imprints on days 3–6 of the primary response would suggest that cellular division becomes more prevalent.

The timing of the changes attributed to lymphocyte transformation is such that it is not possible to differentiate the immunoblast derived by reticular cell activation from the immunoblast derived by small lymphocyte activation. A number of investigators have demonstrated the transformation of small lymphocytes to blast cells, and implicated the small lymphocyte in the formation of the antibody forming cells (Gowans, 1962; Zlotnick, 1963; Langevoort, 1963). The work of Nossal and Mäkelä (1962) suggests that an individual cell can pass through a stage of 19S antibody synthesis prior to the development of 7S antibody forming potential, at least *in vitro*, although the origin of such cells was the resting reticular cell. From the present work, it is believed that the cells which are derived from small lymphocytes by activation in the primary response do synthesize 19S antibody initially and subsequently become 7S antibody forming cells. Such a transformation commences on day 4 of the primary immune response in mice, following which the population of 7S antibody forming cells is increased from days 4 to 6 by cellular proliferation. The most prominent immunogenic cell on day 6 exhibits the morphological features of a plasma cell. Thus, it is concluded that 7S antibody forming cells in the primary response arise from small lymphocyte precursor cells, which, during the process of transformation, pass through a transient stage of 19S antibody production, and subsequently divide to give rise to a population of 7S antibody forming plasma cells.

Several observations may be cited in support of the view that 7S antibody forming cells arise by a process of cellular division. The work of Sahiar and Schwartz (1965), utilizing cytotoxic agents which interfere with proliferation of cells, suggests that the

inhibition of 7S antibody synthesis is mediated by the effects of these agents on the mitotic process. Smith and Robbins (1965) demonstrated that lethal irradiation abolishes the capacity for 7S antibody synthesis presumably by suppressing mitotic activity. Previous work (Eidinger and Pross, 1967) on the cytodynamics of the production of plaque forming cells also implicated cell division in the development of 7S antibody forming cells while 19S antibody forming cells arose by a process of transformation.

These differences in the biological characteristics defining 19S and 7S antibody formation provide strong evidence for the origin of these two classes of immunoglobulins from two populations of cells at any point in time. It is conceivable that a cell might alter from one which forms 19S antibody to one which forms 7S antibody. The work of Nossal and Mäkelä (1962) supports this view. The finding of allotype specificity under the control of the *a* locus, which codes for part of the heavy chains of IgM, IgA and IgG immunoglobulins in all three classes of antibodies in rabbits (Oudin, 1966), also suggests that the potential for formation of all three types of heavy chains may be present in all immunologically competent cells. Thus, while genotypic potential may be present in all cells, the mechanism which governs phenotypic expression of the immunoglobulin class produced during the various phases of the immune response may be turned on by extrinsic controlling factors such as amounts of antibody or antigen in the environment. These variations in phenotype might well be associated with similar morphological variations, which could account for the controversies regarding the origin and function of 19S and 7S antibody forming cells.

The observation in the present work of a morphological sub-type of plasma cell associated with the late phase of the primary immune response, but also occurring in the secondary response, raises the possibility of the existence of morphological subgroups of cells in immunized animals. The association of a plasma cell type with production of a circulating non-IgM, mercaptoethanol sensitive antibody during the late phase of the primary response (Eidinger and Pross, 1967) is compatible with the view that additional distinctive morphological types of cells may be responsible for the production of different immunoglobulins. If 'late' antibody is attributable to the presence of very small numbers of these cells, the persistence of maximal circulating antibody levels suggest that these cells have a longer lifespan and an increased rate of biosynthesis of antibody.

Morphological evidence alone cannot be taken as absolute proof of the sequential changes in the production of antibody forming cells. There is little controversy concerning the complexity of the cellular interactions which constitute the primary immune response. However, morphological studies have a place in defining the origin and activity of antibody forming cells, in providing a basis for the further analysis of the cellular events utilizing radioactive tracers to label precursor cells at appropriate intervals.

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