ANTIGENS IN IMMUNITY

IX. The Antigen Content of Single Antibody-Forming Cells*

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Plate 76

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The purpose of the present series of papers (1–9) is to gain insight into the mode of action of antigenic molecules by a critical study of their distribution through the body. The model system we have used is the injection into the foot-pads of rats of *Salmonella* flagellar antigens labelled with ^{125}I, followed by autoradiographic study of the draining lymph nodes. Key features of experimental design have been the use of (*a*) the minimal amount of antigen capable of achieving the desired effect, be it a primary response, a secondary response, or immunological tolerance; and (*b*) a labelling technique using carrier-free preparations of iodide so that essentially every molecule of antigen is labelled.

Autoradiographs of lymph node sections from animals killed while forming large amounts of antibody showed that two systems of macrophages, namely those in lymphoid follicles and those in medullary sinuses and cords, trapped and retained antigens. In many sections, sheets of plasma cells could be seen with no labelled macrophages in the immediate vicinity; such plasma cells were unlabelled. In other areas, plasmablasts and plasma cells lay close to regions of heavy antigen deposition. As resolution with ^{125}I is by no means perfect, plasma cells close to macrophages frequently had several grains overlying them. It was then impossible to determine whether the disintegrations giving rise to these grains had occurred in the plasma cell itself or in an adjacent macrophage. Moreover, as all adult lymph nodes contain some plasma cells, one could not be certain merely by inspecting sections that any given plasma cell had been forming antibody to the injected, labelled antigen. Thus, while the findings suggested that plasma cells contained little or no antigen, further work on the question was needed.

The basic questions we desired to answer were: (*a*) during induction of antibody formation, does antigen or fragmented antigen enter the precursors of plasma cells, and, if so, how much enters? (*b*) once the cellular events leading

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to antibody formation have been induced in a cell, must antigenic determinants persist in all its progeny as long as they continue to form antibody?

For a variety of reasons, mainly technical, progress in the study of the first question has been slow. The second question has been more amenable to effective examination. In our system very small doses of antigen lead to the formation of 7S antibody without a precedent 19S phase. One week or later following primary stimulation with 0.1 μg or less of flagella or flagellin, cells making 7S antibody were isolated, tested singly for antibody production to these antigens and then processed individually for autoradiography to measure their content of ^{125}I. The study showed that such cells contained less (and perhaps much less) than three or four molecules of macromolecular antigen.

Materials and Methods

Animals.—Wistar albino rats, of both sexes, randomly bred at the the Walter and Eliza Hall Institute, aged 12 weeks at immunization were used. They were fed on "barastoc" dog cubes, cabbage, and tap water.

Antigens.—The preparation and properties of flagella particles and soluble flagellin, from *Salmonella adelaide* have been fully described elsewhere (1). These antigens, after labelling with ^{125}I, were injected into both hind foot-pads or intravenously, on a single occasion, the total dosage being 50 or 100 ng, except in one experiment where 2 μg was used.

Iodination Procedures.—The method of Greenwood, Hunter, and Glover (10), slightly modified as previously described (3) was used. The isotope was carrier-free ^{125}I from the Radiochemical Centre, Amersham, England. Substitution rates varied from 0.1 to 2.1 gm atoms of iodine/30,000 gm of antigen, (specific activities 7 to 140 μc/μg).

Serological Techniques.—All rats were killed by exsanguination under ether anesthesia and serum samples were assayed for content of anti-H antibody, using a serial 2-fold titration method (1, 2). The antibody levels found in rats injected with ^{125}I-labelled antigens were all consistent with those previously reported for equivalent doses of unlabelled antigens (2, 7).

Single Cell and Autoradiographic Techniques.—Our basic technique for studying antibody formation by single cells and performing autoradiography has been fully reported elsewhere (11). Only those aspects specific to the present experiments will be described in detail. Popliteal and aortic lymph nodes, or after intravenous immunization, the spleen, were removed and immediately placed into cold modified (12) Eisen's medium containing 100 ng/ml of flagellin. This carrier antigen was added to minimize the possibility of labelled antigen released from macrophages during teasing of the nodes becoming adsorbed onto the surface of antibody-forming cells through an antigen-antibody reaction. Single cells were released from the tissue by gentle teasing with needles. Then the cells were washed three times with cold medium still containing the carrier antigen. Finally, they were resuspended in the medium without antigen at a concentration of about 5×10^6 cells per ml.

Next, de Fonbrune paraffin-sealed micromanipulation chambers (11) were prepared containing many droplets of medium and a large droplet of the cell suspension. To this droplet was added a droplet containing highly motile *S. adelaide* bacteria. Antibody-forming cells were identified under 200-fold magnification (phase contrast) because they became surrounded by a corona of adherent, partially immobilized bacteria. Previous work has shown that in our system such cells are truly antibody formers and not merely cells covered with cytophilic antibody (12). With a Leitz micromanipulator and a hand-drawn micropipette (orifice *ca.* 10 μ), positive cells were removed singly from the depot droplet and washed 3 to 4 times in clean droplets of medium. This was done by placing the cell into a clean washing

droplet and creating a negative pressure within the micropipette capable of drawing the cell a considerable distance through the droplet. We attach great importance to this manoeuvre. It not only diluted out any free labelled material which the depot droplet might still have contained, but subjected the cell to a mild shearing stress which could have removed lightly adsorbed material; *e.g.*, cell debris. After washing, the cell was placed on a marked area of a gelatin-coated microscope slide. This was then dipped in fixative (89 per cent methanol, 1 per cent acetic acid, 10 per cent H_2O) for 30 minutes, washed in running tap water for 30 minutes and processed for autoradiography using the technique of Messier and Leblond (13). The

TABLE I

Numbers of Grains over Single Antibody-Forming Cells

Antigen used: ^{125}I-flagellin.
^{125}I substitution rate: 2.1 per molecule (assuming mol wt = 30,000).
Time killed after injection: 30 days.
Percent ^{125}I atoms disintegrating during time of exposure of autoradiograph: 35 per cent.
Organ used: popliteal lymph node.

Cell No.	No. of grains over cell	No. of grains over equal background area	Net grain count/cell
1	0	2	−2
2	4	3	1
3	1	3	−2
4	0	0	0
5	3	2	1
6	0	0	0
7	1	1	0
8	0	0	0
9	1	0	1
10	2	4	−2
11	0	0	0
12	0	2	−2
13	0	0	0
14	1	2	−1
15	0	0	0
16	0	1	−1

slides were dipped in kodak NTB/2 photographic emulsion and allowed to dry in a horizontal position NTB(H). Autoradiographs were exposed for 60 days, *i.e.*, just over one half-life (58 days) of the isotope. After standard development and fixing, they were stained with Giemsa and examined at 1250-fold magnification. The numbers of grains overlying each cell and an equal area of background in the same microscopic field were recorded.

Preparation of Sections.—Histological sections, 5 μ thick, were prepared from spleens and aortic nodes, and occasionally from portions of the popliteal node by conventional techniques (formalin fixation, paraffin embedding). These slides were dipped in NTB/2 emulsion and allowed to dry in a vertical position, NTB(V). They were exposed for 60 days, developed, and stained with methyl green-pyronin. The most heavily labelled follicular area and medullary region were located, and grain counts performed. With the aid of an eyepiece graticule, 100 areas measuring 4 × 4 μ each were counted; background over an equivalent area was sub-

tracted yielding a net count. As these counts represent maximally labelled areas, they are not directly comparable with those reported previously, where areas with apparently median degrees of labelling had been scored.

Number of Grains Per Disintegration of ^{125}I.—In a separate study (14), figures have been obtained for the mean number of grains resulting in various photographic emulsions following the disintegration of a single atom of ^{125}I. First, iodinated erythrocytes, or erythrocytes to which iodinated protein had been conjugated, were exposed to different photographic emulsions, and, after a suitable exposure time, the number of grains overlying cells were scored. In this situation, however, there was uneven distribution of isotope. One-half of the isotope (on the upper surface of the flattened erythrocyte) was in intimate contact with the emulsion; the other half, with the slide. To correct for this, protein films of various thicknesses were made in which iodinated protein was uniformly distributed throughout the film. It was practicable to perform this study with only one emulsion. The value for number of grains per disintegration was about half of that obtained in the red cell experiments, but for reasons discussed fully elsewhere (14), is regarded as representing a more valid figure. Over the range of film thickness 0.5 to 5.0 μ, the value was essentially constant, and was calculated to be 0.23 grains per disintegration using NTB(V) and 0.36 using NTB(H). These values have been used in the interpretation of the present experiments.

TABLE II

Antigen Content of Single Antibody-Forming Cells: Summary of Results

Experiment No.	Labelled antigen injected	Antigen dose	Iodine substitution rate	Time killed after antigen injection	Organ studied	No. of antibody-forming cells scored	Mean net grain count per cell	Mean net grain count expected for 10 molecules/cell
				days				
1	Flagella	100 ng	0.6	7	A*	18	−0.1	1.0
2	Flagella	100 ng	0.6	14	P	14	0.4	0.9
3	Flagella	100 ng	0.6	43	P	14	0.3	0.6
4	Flagellin	100 ng	2.1	29	P	13	−0.5	2.6
5	Flagellin	100 ng	2.1	30	P	16	−0.4	2.6
6	Flagellin	100 ng	2.1	35	P	17	0.0	2.5
7	Flagellin	100 ng	2.1	42	P	17	0.1	2.3
8	Flagellin	100 ng	2.1	44	P	13	−0.2	2.2
9	Flagellin	100 ng	2.1	48	P	10	−0.1	2.1
10	Flagellin	100 ng	2.1	51	P	7	−0.4	2.1
11	Flagella	2 μg	0.1	7	P	21	−0.1	0.2
12	Flagella	50 ng	1.2	39	S	15	0.0	1.3
13	Flagella	50 ng	1.2	40	S	15	0.1	1.3
14	Flagella	50 ng	1.2	60	S	12	0.0	1.0
15	Flagella	50 ng	1.2	66	S	14	0.0	1.0

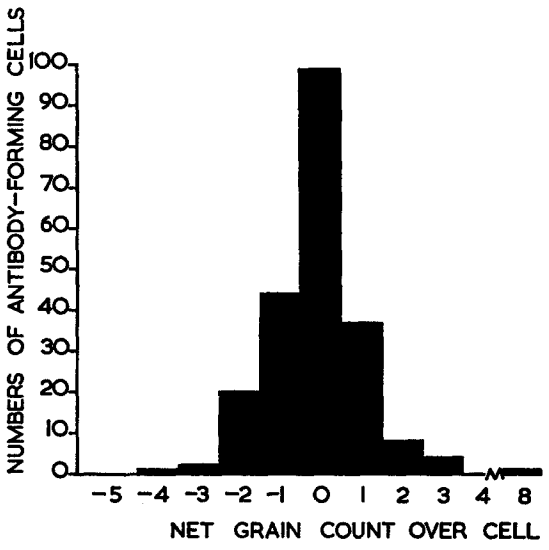
* A denotes aortic node; P, popliteal node; and S, spleen.

RESULTS

Single Cell Studies.—The results from a typical experiment using 100 ng of ^{125}I flagellin are presented in Table I. In this rat, the original level of iodination

of the labelled antigen at the time of injection had been 2.1 atoms ^{125}I/molecule, but as 30 days elapsed between time of injection and time of killing, 30 per cent of the ^{125}I atoms would have decayed during the animal's life. Exposing autoradiographs for one half-life thus resulted in one-half of 70 per cent, or 35 per cent, of the original ^{125}I atoms decaying over the period that the autoradiograph had been exposed.

The assumption that the distribution of ^{125}I in our experiments is a valid reflection of the distribution of antigenic material generally will be discussed below. Using this assumption, we can calculate that if one molecule of the



TEXT-FIG. 1. Frequency distribution of net grain counts over all cells studied.

original antigen had been present in the antibody-forming cell at the time of killing of the animal, and had been adequately fixed by the methanol-acetic acid mixture, the cell would have given a net grain count of $0.36 \times 35/100 \times 2.1$ or 0.26 gr. In fact, the mean net grain count was -0.4, there being actually fewer grains over the cells than over the background.

The results of 216 antibody-forming cells from 15 separate experiments (including the above) are presented in Table II. Included in this number were all the different categories of antibody-forming cells; plasmablasts, immature and mature plasma cells, and lymphokinecytes (Figs. 1 *a* to 1 *d*). Negative mean net grain counts over antibody-forming cells were observed in somewhat more experiments than positive means. Net grain counts over individual cells appeared to follow a normal frequency distribution around a mean value near zero (Text-fig. 1). These findings strongly suggest that the silver grains found

over certain antibody-forming cells represented background, and not the presence of ^{125}I in the cell. A single cell with a net grain count of 8 may or may not have represented an exception.

Grain Count Determinations on Sections.—Results of grain counts performed on sections from some antibody-forming organs are presented in Table III. Though many thousands of plasma cells were scanned in these sections, no unequivocally labelled ones were encountered. Moreover, even the phagocytic cells of lymph nodes and spleen showed little label. In experiments 12 and 13, for example, we had to search extensively to find any area with detectable label, spleen sections in general showing somewhat more heterogeneity in

TABLE III

Number of Antigen Molecules per 4 × 4 μ‡ Area of Sections of Antibody-Forming Organs*

Experiment No.	Time killed after antigen injection	Organ§ studied	No. of antigens molecules in follicle	No. of antigen molecules in medulla or red pulp
	days			
2	14	A	110	120
3	43	P	200	180
4	29	P	4.1	3.6
5	30	P	8.1	—
8	44	P	2.3	7.0
10	51	P	1.5	3.6
12	39	S	6.2	17
13	40	S	3.3	6.5

* Assuming ^{125}I distribution reflects antigen distribution.

‡ One area 4 x 4 μ encompasses *ca.* 1 small lymphocyte. Four such areas cover 1 typical medullary macrophage.

§ A, aortic node; P, popliteal node; S, spleen.

labelling intensity from field to field than lymph node sections. In experiments 4 to 10, even the most heavily labelled medullary regions suggested the presence of only 14 to 28 antigen molecules per macrophage. A typical medullary area with labelled macrophages and unlabelled plasma cells is shown in Fig. 2.

DISCUSSION

The present experiments were designed to assess the antigen content of antibody-forming cells found at relatively late stages of the 7S antibody response to minute doses of flagellar antigens. The reason that this phase of antibody production was first subjected to detailed study is that antibody levels at this time are much less dependent on antigen dose than during the early, inductive phase (2, 7). Thus, small amounts of radioactivity were involved and the lymphatic tissues at the time of killing contained little isotope. Conse-

quently it was not difficult to remove radioactivity from the supernatant of cell suspensions by washing, and background grain development unassociated with cells was not a problem in the autoradiographs of single cells. The study showed that even when flagellin containing an average of more than one ^{125}I atom per molecule was used as antigen, the antibody-forming cells were, on the average, no more heavily labelled than was the background.

Taken at face value, the results suggest that antibody-forming cells at this stage of the immune response contain no antigen. It is essential to consider carefully the various assumptions which must be made before this result can be accepted.

1. Deiodination of Macromolecular Antigen.—During iodination of flagella or flagellin, iodine reacts with tyrosine residues in the protein. Does iodine become detached from the protein while in the lymph node? Although there is little evidence from work with other proteins that this occurs, proof that it does not occur has been lacking. Work carried out in conjunction with this study (Ada, data, in preparation) has shown that even up to 2 months after injection of labelled flagella and for shorter periods with labelled flagellin, most of the label in a lymph node extract is firmly bound to granular material and, when extracted, can be shown to be associated with material which will react with specific antiserum. Thus, deiodination of the antigen while in the macromolecular state is not a major worry.

2. The Question of Active Fragments of Antigen.—Antigens are degraded in the body and it is possible that a degradation product, rather than the intact antigen, may act as the inducer of antibody formation. While there is evidence that deiodination of the macromolecule does not occur to an appreciable extent, we do not know whether it might occur more readily in the case of an oligopeptide containing iodotyrosine. If it does, our studies could obviously not detect deiodinated active fragments. If it does not, the question still remains of how many of the amino acids in the flagellin molecule (*ca.* 290) are concerned in the antigenic site(s). Flagellin contains 4 tyrosines and in most molecules a maximum of 2 could be iodinated by our procedure. It has recently been shown (15) that the flagellar antigen, i, consists of at least 13 separable subfactors and possibly many more. The antigen fg may be equally as complicated. Whether tyrosine is present in any of the antigenic determinants remains to be determined; *e.g.*, by a study of protein fragments obtained by *in vitro* degradation procedures. Some uncertainty must also exist as to whether a small fragment could be lost during isolation and fixation of cells. This may not be a serious risk as only viable cells were isolated for study and the fragment might well be associated with a macromolecular structure in the cell. Obviously clear-cut answers to these questions cannot at present be given.

We must consider whether additional approaches might have yielded more clear-cut answers about the fate of antigen or antigenic fragments than the

use of the "external" label, ^{125}I. It has already been stressed (3) that in order to detect small numbers of molecules in this type of study, an isotope of relatively short half-life must be used. Thus, introduction of ^{3}H or ^{14}C by biosynthetic incorporation could never lead to sufficiently high specific activities of antigen for studies where meaningful *negative* answers are to be obtained. Biosynthetic labelling with ^{35}S remains a possibility; specific activities obtained would probably not be as high as those obtained in the present work, but, by virtue of the different amino acid residue which would become labelled, complementary information on the fate of antigenic fragments might be obtained.

The Number of Antigen Macromolecules in Antibody-Forming Cells.—This study has placed an upper limit on the number of antigen macromolecules which must be present in the mature, antibody-forming cell. In the experiments with the most heavily labelled flagellin, a mean antigen content of 4 molecules per cell would have yielded a net mean grain count around 1 (Table II), and in fact the observed counts did not differ significantly from 0.

In view of the rather high background grain development observed with NTB(V), we are disinclined to attach importance to the one cell with a net grain count of 8, though this might well have been a cell with antigen in or on it. In most cells, there must have been less than 5 molecules of intact antigen, and there may well have been none at all, a concept supported by the slight negative net mean grain count over all cells included in the study. Globulin is formed on polysomes (16) and, though we do not know how many such units were actively synthesizing antibody in our cells, it seems likely that the number was large. Thus, it seems highly improbable that an antigen macromolecule, acting as a direct template, was present on each synthetic unit.

The failure to find antigen in plasma cells conflicts with two recent studies (17, 18). In the first (17) Roberts injected ^{3}H-labelled azoprotein antigens into mice, and found some labelled plasmablasts and plasma cells. Interpretation of this study is made difficult by the fact that numerous injections of labelled material were given. It was thus not possible to know whether the antigen in or on plasma cells had played an inductive rôle, or had merely come into association with the cell after antibody production had been established. In the second (18) Wellensiek and Coons followed the spread of ferritin through the lymphatic system of the rabbit by immunofluorescent and electron microscopic techniques. In the primary response, *no* antigen could be found in plasma cells. In the secondary response, both plasmablasts and plasma cells regularly contained ferritin, the former in greater amount than the latter. It was estimated that mature plasma cells contained up to 12,000 antigen molecules. We have not yet analyzed the antigen content of plasma cells in the secondary response in detail, but inspection of sections shows that it must be very low. Part of the great difference in antigen content between these experiments and our own might be associated with the difference in antigen dose.

Wellensiek and Coons used 100,000 times more antigen than was involved in the present study.

It remains to be seen whether macromolecular antigen enters plasmablasts during the induction phase of antibody formation, but does not persist in the progeny of the precursor cells. This, and the question of antigen fragments in cells, is under study.

SUMMARY

Flagellar antigens from *S. adelaide* bacteria were labelled with carrier-free ^{125}I so as to achieve a substitution rate of 0.1 to 2.1 radioactive iodine atoms per flagellin molecule. Lymph nodes from rats injected with small amounts of these antigens were teased into a single cell suspension. Single antibody-forming cells were identified and submitted individually to autoradiography so as to measure their content of iodine ^{125}I. The study was confined to the 7S phase of the primary response.

Grain counts over 216 single antibody-forming cells were no higher than counts over equivalent background areas in the emulsion. This finding suggested that the cells contained little or no macromolecular antigen, and it was considered very unlikely that there were sufficient macromolecules of antigen in a plasma cell to act as a direct template on polysomes for the formation of antibody. The question of the possible presence, in such cells, of fragments of antigen was considered. This possibility, while not supported by the present results, cannot be excluded at present.

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EXPLANATION OF PLATE 76

FIGS. 1 *a* to 1 *d*. Autoradiographs of single antibody-forming cells from rats injected with ^{125}I-labelled flagellar antigens. NTB/2, exposure 60 days, *ca.* × 2000.

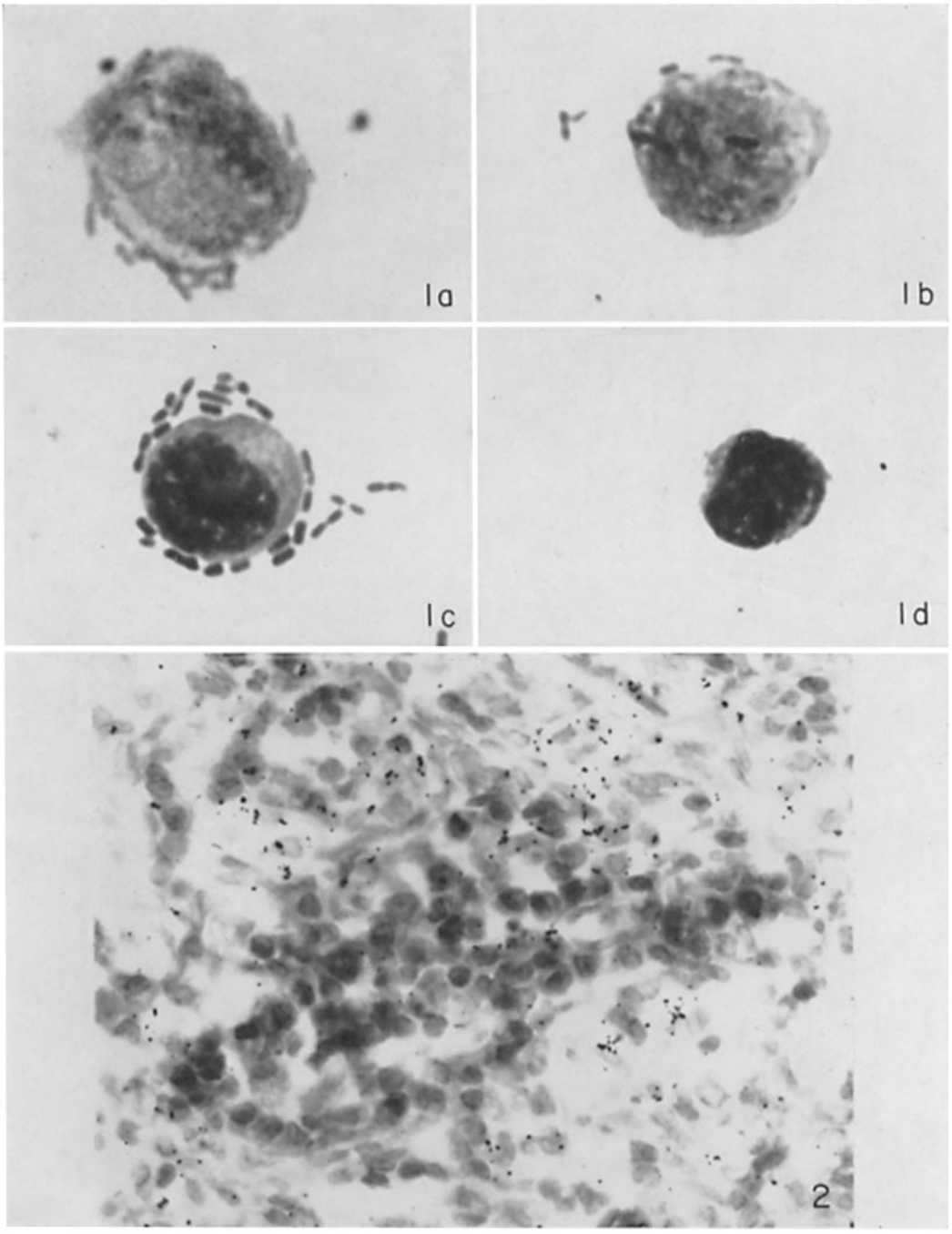
FIG. 1 *a*. Plasmablast.

FIG. 1 *b*. Immature plasma cell.

FIG. 1 *c*. Mature plasma cell.

FIG. 1 *d*. Lymphokinecyte.

FIG. 2. Autoradiograph of medullary region of section of popliteal lymph node from rat used in experiment 8. Note the light labelling of macrophages and the absence of detectable labelling in plasma cells. NTB/2, exposure 60 days, × 500.



(Nossal *et al.*: Antigen in single cells)