THE MECHANISM OF ACTION OF ANTI-LYMPHOCYTE SERUM

STUDIES OF ANTIBODY ELUATE*

BY EUGENE M. LANCE,‡ M.D.

(From The National Institute For Medical Research, Mill Hill, London, England)

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Although the antibodies responsible in vivo for the immunosuppressive action of anti-lymphocyte serum (ALS) are of IgG specificity (1, 2), only a small fraction of the total IgG is effectively anti-lymphocytic. Lance (2) and Woodruff et al. (3) have estimated by absorption studies of radioactive ALS IgG that the relevant antibodies comprise between 1–5% of the total serum IgG. The importance of this observation becomes apparent when attempts are made to trace the fate of the active component, since the results may well by obscured by the 95% IgG irrelevant to the action of ALS. In order to study the natural history of the relevant molecules as a means of gaining insight into the mechanism of action of ALS, it was deemed necessary to enrich the specificity of the preparation. In this communication the method used to achieve this end and the results of study of the product obtained will be described.

Materials and Methods

Animals.—Mice were obtained from the breeding unit at the National Institute for Medical Research, Mill Hill, London, England, and included the inbred strain of CBA and outbred strains of Tyler, VS, or Parkes mice. The outbred animals were used as a source of thymus in the production of ALS.

Preparation of ALS.—Adult New Zealand White rabbits received two intravenous injections of 1×10^9 mouse thymocytes 2 wk apart. 1 wk later they were exsanguinated from the heart and the pooled serum was inactivated at 56°C for 30 min, sterilized by Seitz filtration and stored at −20°C until use (4).

Isolation of IgG.—The crude globulin was extracted from samples of whole serum by ammonium sulfate precipitation (5) and after equilibration with the appropriate buffer, the IgG component was isolated by column chromatography on DEAE-cellulose[1] (6). Protein concentrations were determined by spectrophotometry with a Unicam spectrophotometer reading at 280 mμ (extinction coefficient for gamma globulin = 14 for a 1% solution). The purity of the preparations were checked by immunoelectrophoresis (7) using goat anti whole rabbit serum and goat anti rabbit IgG Fc piece (kindly supplied by Dr. Sayaka Utsumi).

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‡ Present address: The Hospital for Special Surgery, 535 East 70th Street, New York, N.Y.

[1] DEAE-cellulose, diethylaminoethyl cellulose.

Iodination of Proteins.—Proteins were labeled with either carrier free ^{131}I or ^{125}I (Radiochemicals-Amersham, England) by the method of McFarlane (8).

Preparation of Eluate.—The general scheme was to cause samples of ALS IgG to react with acid-washed "membrane" preparations of thymocytes, to elute the "anti-lymphocytic" antibody in the presence of acid pH and to recover the enriched product (ELX) after passage through DEAE-cellulose. A stepwise description of the procedure will be followed by a typical protocol.

1. *Preparation of Thymocyte "Membrane".*—Initially, whole thymocytes were used to absorb ELX from ALS IgG. However, it was found that the acid elution step released great quantities of extraneous cell material into the supernatant, thus making subsequent isolation of IgG more difficult. Formalized cells had the disadvantage of increasing the nonspecific absorption. A membrane preparation ("crude insoluble" lipoprotein fraction) (9) of mouse thymocytes proved an efficient absorbing material but again released troublesome material into the eluate. Pretreatment of the "membrane" with 0.1 M citric acid (pH 2.5) for 60 min largely eliminated this difficulty, although some loss of absorbing ability was associated with this step. The acid washed membrane could be stored at 4°C under strong salt (3.0 M) or at −20°C, and preserved absorption potency.

2. *Reaction of Membrane with ALS IgG.*—The ALS IgG preparations were sometimes trace labeled with ^{131}I during the initial runs when optimum conditions were being sought. The final protein concentration was adjusted to 2 mg/ml and the ALS IgG and membrane were mixed at a ratio of between 0.5 to 1.0 mg of IgG per membrane yield of 10^9 thymocytes. This ratio provided maximum specific binding and allowed final recovery of ELX representing about 2% of the original IgG sample. After incubation in a water bath for 30 min at 37°C the mixture was spun at 25,000 rpm in a model L Spinco Ultracentrifuge using an SW 25 rotor for 1 hr. The supernatant was discarded (or portion kept for determination of residual radioactivity) and the sediment washed 3× in phosphate buffered saline (PBS), pH 7.2, by resuspending in a hand homogenizer with interval centrifugation as above.

3. *Elution Step.*—After the last spin the sediment was resuspended by hand homogenization in approximately 40 ml of 0.1 M citric acid (pH 2.5) and placed at 4°C with constant magnetic stirring for 30 min. The mixture was then centrifuged as above and the supernatant collected. (In those experiments where the method was evaluated, samples of the supernatant and sediment were counted for residual radioactivity.) The eluate was placed in Visking dialysis tubing and pressure dialyzed against PBS (pH 7.2) to equilibrium and to a final volume of about 5 ml. Initially the eluate was neutralized by the dropwise addition of 0.5 N NaOH, but it is our impression that denaturation was greater than through the slower neutralization achieved by dialysis. After neutralization a small amount of precipitate was usually noted and was removed by centrifugation.

4. *Recovery of IgG molecules from Eluate.*—The neutralized eluate was dialyzed against 0.02 M phosphate buffer (pH 8.0) overnight and then placed on a DEAE-cellulose column and the IgG molecules eluted with 0.02 M phosphate (pH 7.4). The final eluate was concentrated by pressure and dialysis against PBS (pH. 7.2), sterilized by Millipore filtration, and stored at −20°C. The protein concentration was determined by spectrophotometry.

Protocol.—1. The membrane fraction was prepared from thymocytes of 500 mice (wet weight 30.4 g) and exposed to 0.1 M citric acid (pH 2.5) for 60 min. The sediment was washed 2 × in PBS.

2. 5 mg of ALS IgG were trace-labeled with ^{131}I (100 μc with 50% efficiency) and added to 45 mg of ALS IgG at a final concentration of 2 mg/ml, i.e., 25 ml total volume. A 0.1 ml portion was set aside as a standard.

3. The acid washed membrane and the ALS IgG solution were then thoroughly mixed and placed in a 37°C water bath with agitation for 30 min. The sediment was washed 3 × in PBS.

4. The sediment was resuspended in 40 ml of 0.1 M citric acid and stirred in the cold for 30 min. After centrifugation the sediment and the supernatant were collected. The supernatant was pressure dialyzed against PBS overnight to a final volume of 10 ml with the formation of a slight precipitate. The sample was centrifuged at 3000 *g* and the precipitate and supernatant collected. At this stage the radioactivity in the eluate supernatant indicated that it contained 1.3 mg of IgG but the optical density at 280 mμ indicated that the total protein content was about 2.4 mg. Therefore, approximately 1.1 mg of contaminating protein was present which had been released from the membrane substance itself.

5. The eluate was equilibrated against 0.02 M phosphate (pH 8.0) and passed through a DEAE-cellulose column using 0.02 M phosphate pH 7.4 buffer. The column consisted of a 10 ml plastic syringe containing 4 ml of DEAE-cellulose (Whatman Chromedia DE-32). 100 drop

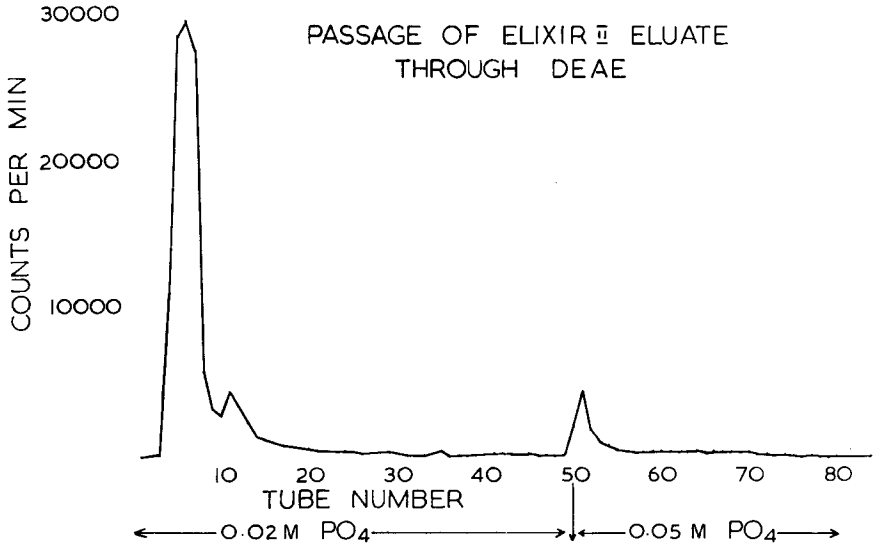

FIG. 1. See text for explanation

samples were collected and the concentration of IgG was monitored by determing the radioactivity in the fractions (Fig. 1). After 50 tubes had been collected, the buffer was changed to 0.05 M phosphate and an additional 35 tubes collected. This ensured that all the protein had been removed from the column. Tubes 4–10 containing the first peak were pooled and pressure dialyzed versus PBS to a final volume of 3.0 ml.

6. Counting the original standard, the sediment membrane, the precipitate, and the final ELX preparation for residual radioactivity allowed calculation of the percentage of IgG to be found at various stages. In this run, the final recovery of ALS IgG as ELX was 1.9%. The amount of protein bound to membrane but not eluted was 1.0%, and the amount of protein lost as precipitate after neutralization of eluate was 0.2%. Small amounts of IgG were also, of course, lost on the column and in the discarded fractions eluted from DEAE. The total protein contained in the final preparation was 1.0 mg, as estimated by optical density measurement (2.0% of the original sample) and this excellent agreement between the determinations by radioactivity and optical density indicates that very little if any contaminant was present in the final preparation.

RESULTS

Characterization of ELX.—1. Immunoelectrophoresis of the enriched eluate (ELX) solutions showed a single arc of precipitation in the IgG region. The ELX appeared to be a more homogeneous substance as compared with the arc found against whole ALS IgG (Fig. 2).

2. Samples of ELX were run on a sucrose gradient comparing the migration of the trace-labeled ELX molecules with those of native rabbit IgG. The results are shown in Fig. 3. 20 μg of trace labeled ^{131}I ELX was added to 1.0 mg. of normal rabbit IgG to which some mouse hemoglobin was added. This sample was placed on top of a discontinuous sucrose gradient which had been prepared by

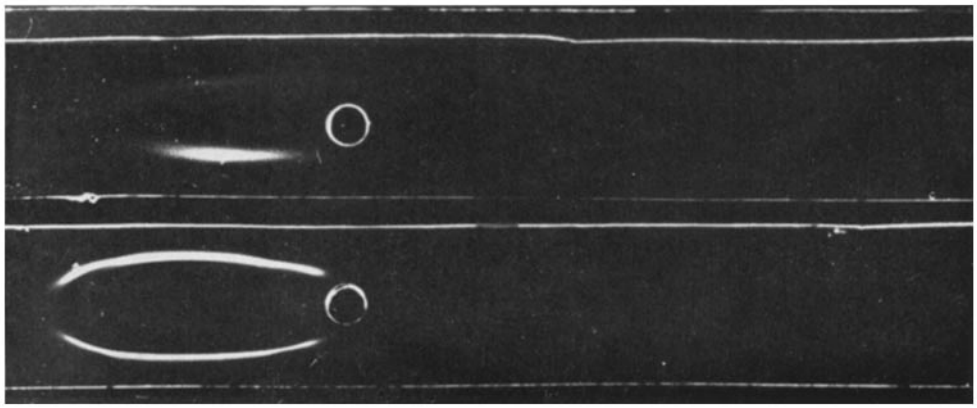

FIG. 2. Immunoelectrophoresis of ELX (top well) and ALS IgG (bottom well) against goat anti whole rabbit serum (top and bottom trough) and goat anti rabbit gamma G (middle trough). The ALS IgG molecules appear to have a broader range of electrophoretic mobility than that found in the ELX subcomponent.

layering consecutively 40%, 25%, and 10% sucrose in volumes of 1.5 ml upon another. The plastic tube containing the gradient and sample was spun for 18 hr at 22°C in a model L Spinco ultracentrifuge at 35,000 rpm using an SW 39 rotor. Fractions of eight drops were collected from the bottom of the gradient and each fraction counted for residual radioactivity and the optical density at 280 and 575 mμ determined. The peak of radioactivity coincided fairly well with that for IgG and was distinct from that of hemoglobin indicating that no gross denaturation was present.

3. In vitro binding to lymphocyte membrane: 60 μg of ^{131}I trace labeled ELX containing 133,000 counts per minute was allowed to react at 37°C for 30 min min with 1.5×10^9 thymocytes prepared from the thymuses of 10 A strain mice. After centrifugation, 40% of the counts remained in the supernatant. A second absorption with 1×10^9 cells was carried out in a similar manner and the

second supernatant contained 25% of the original counts. Therefore, with two exposures, 75% of the molecules were removed from solution. Control experiments in which equivalent amounts of NRS IgG molecules were subjected to similar numbers of cells never gave more than 4–5% cumulative absorption. Therefore, the excess absorption of ELX molecules can be attributed to reten-

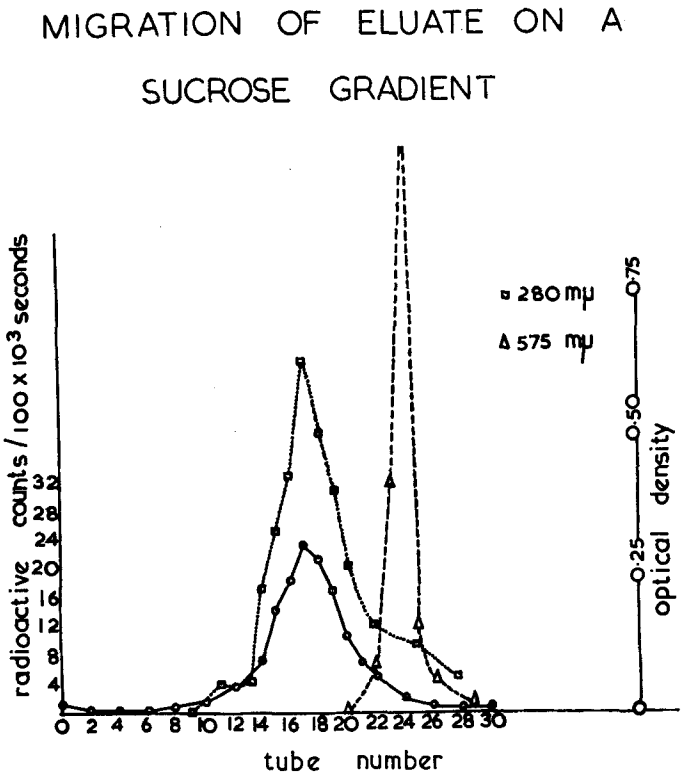

FIG. 3. The open circles represent the distribution of radioactivity in the gradient which coincides with the reading of OD at 280 mμ. See text for details.

tion of antibody affinity for lymphocyte receptors. In retrospect, the amount of antigen provided was too low for the quantity of antibody to expect maximum absorption; (the optimum ratio for ALS IgG, roughly 10^9 cells/500 μg, must be multiplied by 30–40 for ELX).

4. The ability of ELX preparations to kill lymphocytes in vitro in the presence of complement was determined by Dr. Marian Ruszkiewicz. When ELX II, at a concentration of 700 μg per ml, was run in serial two dilutions, the cytotoxic end point titer was 1/4096 (Fig. 4). Comparing this to the titre of the parent antiserum XXI ALS IgG, and correcting for the difference in protein concen-

tration we find that gram for gram, the ELX preparation is roughly 10 times more efficient in killing lymphocytes in vitro.

5. Activity in vivo. the studies thus far have shown that ELX is composed of a relatively homogeneous population of IgG molecules which are not denatured to any great extent and which retain the ability to bind to and kill lymphocytes in vitro. However, ELX would prove useful only if it also retained the ability to prolong the survival of skin homografts. To conserve material, the assay used was based upon the studies of Jooste (10) who showed that the survival of skin homografts could be prolonged by the injection of newborn mice with small quantities of ALS whole serum. 10 newborn CBA mice received a subcutaneous

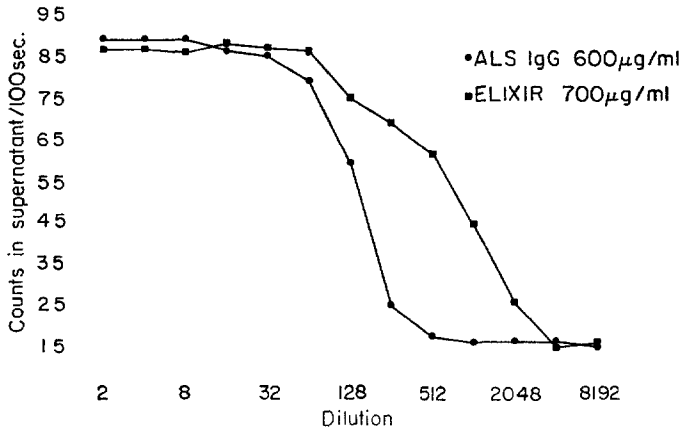

FIG. 4. Comparison of the ability of ELX and ALS IgG to release radioactivity from ^{51}Cr labeled target lymphocytes in the presence of rabbit complement.

injection of 7 μg of ELX on the 1st and 23rd day of life and 17 μg on the 11th day. On the 21st day these mice and five untreated control animals of the same age received an A strain tail skin graft. The survival times of the grafts on controls and ELX-treated mice are given in Fig. 5. The mean expectation of life has been prolonged from 9.5 days ±1.7 to 14.0 ±2.1. The total amount of protein given (31 μg IgG) represents approximately 1/50 of the dose that Jooste used to promote homograft survival in this strain combination in a similar though not identical protocol (10).

6. Controls. Because we were concerned that acid exposure might denature the IgG molecules, two control experiments were performed to determine to what extent the preparation of ELX molecules results in a biologically different product. In the first experiment the effect of acid exposure on the potency of whole ALS was studied. 10 ml of whole ALS were divided into two 5 ml portions and one was dialyzed against 100 vol of 0.1 M citric acid (pH 2.5) for 2 hr, fol-

lowed by dialysis 2× against 1000 volumes of PBS. The other portion was untreated. Two groups of five CBA male mice were grafted with A strain tail skin. The first group received 0.5 ml of the untreated ALS subcutaneously on the 3rd and 5th postgraft day, whereas the second group received an equal quantity of the acid-exposed ALS. A slight but nonsignificant decrease in the mean survival time was noted for the group given acid-treated ALS.

The second control measured the amount of denaturation induced by acid

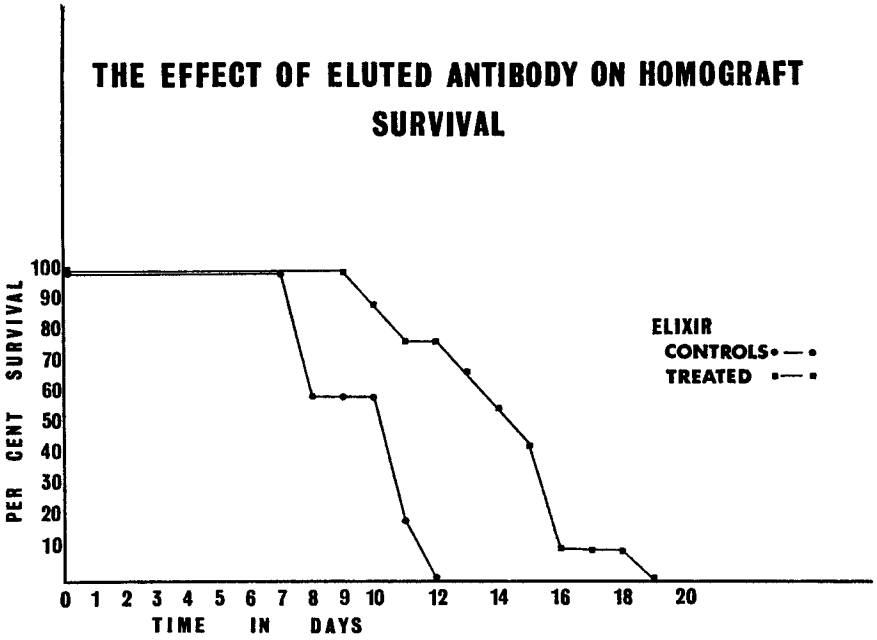

FIG. 5. Comparing the day to day survival of A strain skin homografts on CBA mice which were either untreated (controls) or had received 31 μg of ELX subcutaneously.

exposure through study of the rate of elimination of acid-treated NRS IgG in comparison with untreated NRS IgG. 7.5 mg NRS IgG (1.0 ml volume) was dialyzed vs. 2000 vol of 0.1 M citric acid (pH 2.5) for 12 hr and then against 2000 vol of PBS (pH 7.4) for 24 hr. Samples of NRS IgG and acid-treated NRS were labeled with ^{131}I and injected intraperitoneally into groups of six mice. The whole body counts were recorded daily. The rate of elimination for the two samples was comparable (see Fig. 7, groups A and B). However, after acid exposure a precipitate had formed which was removed by centrifugation. By determination of the protein content before and after acidification, approximately 20% of the protein was grossly denatured. These two controls indicate that there is definite denaturation of protein by the exposure to low pH used in the enrich-

ment procedure. However, even with prolonged exposure (12 hr in second control) a minor fraction is affected, can be removed by centrifugation, and the remainder appears to be handled in vivo normally. The biological activity of the antibody fraction may be slightly reduced by this treatment, but if so, only in a minor way.

Elimination of ELX from Recipient Animals.—The rate of elimination of specific antibody from recipient mice was determined in four separate experiments,

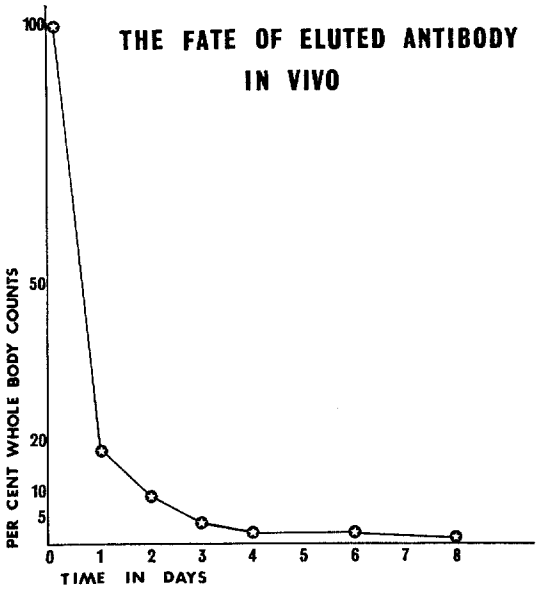

FIG. 6. 16 CBA female mice received an intraperitoneal injection of ^{131}I labeled ELX II (80 μg/animal) and whole body counts were performed at 1 hr and 1, 2, 3, 4, 6, and 8 days thereafter. The results are expressed as the average percentage of label remaining at the various times after appropriate corrections for background and decay. The count at 1 hr is taken as 100%.

using three different lots of ELX (Figs. 6 and 7). In some cases the material was administered subcutaneously; in others, intraperitoneally. Other variations were the inclusion of carrier whole ALS IgG in the inoculum, or in some cases, the prior injection of whole ALS. Controls received both normal and acid-washed NRS IgG injections. On most occasions, iodide water was substituted for the normal drinking water of these animals 24 hr prior to injection. However, on one occasion, the substitution was not performed until the day of injection, with the result that the whole body retention reflected in part thyroid uptake and, therefore, gave somewhat higher values. Regardless of these differences, the trend is very clear. Whereas normal rabbit IgG (and ALS IgG as well) is

eliminated from the body exponentially with a half-life of between 5 and 6 days (roughly equivalent to that found for the elimination from the serum) the ELX molecules are much more rapidly cleared. There appear to be two phases to

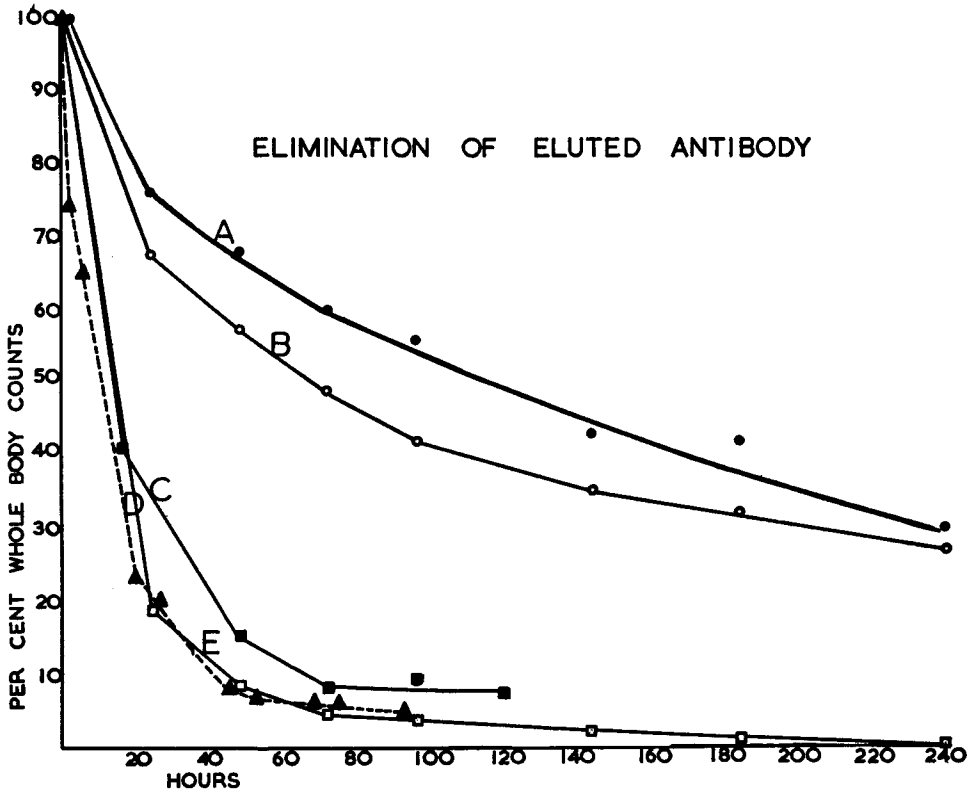

FIG. 7. Each curve represents the average values of panels of 6 CBA females which received: A, ●——●, 400 μg NRS IgG intraperitoneally; B, ○——○, 250 μg NRS IgG intraperitoneally (exposed to acid, pH 2.5 for 12 hr before reneutralization); C, □——□, 300 μg ELX III plus 0.5 cc whole ALS subcutaneously (iodide water started on day of injection); D, ■——■, 50 μg ELX IV intraperitoneally; E, ▲——▲, ELX as group D but 4 hr after 0.5 cc whole ALS subcutaneously. All preparations were labeled with ^{131}I and whole body counts were performed immediately after injection and at the indicated times thereafter. After correction for background and decay, the results are expressed as the average per cent radioactivity remaining at each interval (time 0 = 100%).

this clearance. During the first 24 hr about 70–80% of the molecules are eliminated, and from 24 hr onward the half-life of the remainder appears to be about 24 hr. A small fraction (less than 5%) of the original inoculum may remain within the body for a considerably longer period and about 1–2% may still be detected at the end of 1 wk (Fig. 6).

Distribution of ELX in recipient animals.—Our initial studies attempting to define the tissue localization of anti-lymphocyte antibody compared the distribution of labeled crude gamma globulin (GG) fractions (1.75 M ammonium sulfate precipitation) of NRS and ALS. 60 μg of ^{131}I labeled ALS or NRS gamma globulin was injected into two groups of 30 mice subcutaneously in the right axilla. Five mice from each group were killed at 1, 2, 4, 24, 48, and 72 hr post-injection, and the right and left axillary lymph nodes, spleen, thymus, and a

TABLE I

The Distribution in Mice of Labeled Gamma Globulin from ALS or NRS

Time	Percentage uptake in cells				
	R axillary nodes	L axillary nodes	Spleen	Thymus	Serum
hr					
Group *A*					
1	0.69	0.02	0.01	0.01	1.1
2	0.83	0.02	0.03	0.04	2.7
4	0.31	0.02	0.04	0.02	4.9
24	0.16	0.04	0.10	0.05	16.1
48	0.05	0.04	0.09	0.06	12.8
72	0.04	0.03	0.07	0.02	9.6
Group *B*‡					
1	0.25	0.02	0.02	0.01	1.1
2	0.77	0.01	0.02	0.03	2.8
4	0.25	0.01	0.05	0.02	4.2
24	0.12	0.04	0.09	0.06	10.0
48	0.03	0.03	0.06	0.04	11.8
72	0.04	0.03	0.06	0.03	11.5

* Group A consisted of 30 CBA male mice which received at time 60 μg of ALS gamma G labeled with ^{131}I in 0.5 ml of NRS injected subcutaneously into the right axilla. At each time interval, 5 mice were killed and their organs removed for radioactive counting.

‡ Group B was treated in an identical fashion except that they received 60 μg of NRS gamma B labeled with ^{131}I in 0.5 ml NRS.

sample of serum removed for radioactive counting (Table I). No significant difference was discerned between the two groups with the possible exceptions of a greater uptake by the draining (R) nodes at 1 hr and a greater concentration of counts in the serum at 24 hr in animals receiving ALS GG. It is none the less apparent that neither of the two materials localizes in lymphoid organs to any great extent.

In a second experiment of similar design (Table II) samples were collected at 2 and 24 hr chosen to represent the time of maximal uptake in the draining node and the maximum concentration in the blood stream. In this case, however, cell suspensions were made from the various lymphoid organs and the leukocytes

were collected from the peripheral blood by sedimentation in 1.5% gelatin. These cell collections were then washed 3× in saline to get rid of non-cell-bound protein. It was hoped in this way to make obvious differences obscured in the first experiment by the presence of large quantities of protein in the extracellular fluid. In all cases the amount of protein found on the cells is quite small and of comparable orders of magnitude for the two groups with the one exception that at 24 hr there is far more ALS globulin attached to the cells of the draining node. Both experiments indicate that the localization of ALS gamma globulin like NRS gamma globulin is minor in lymphoid organs and that the fate of any specific antibody is obscured by the abundance of extraneous protein.

TABLE II

Distribution of Gamma Globulin from ALS or NRS in Mouse Lymphoid Cells

Time	Percentage uptake in cells			
	R axillary nodes	L axillary nodes	Peripheral leukocytes	Spleen
hr				
Group *A*				
2	0.00	0.00	0.00	0.00
24	0.02	0.003	0.01	0.04
Group *B*				
2	0.008	0.01	0.00	0.00
24	0.00	0.00	0.01	0.03

CBA mice received 60 μg of ^{131}I labeled ALS gamma globulin (group A) or NRS gamma globulin (group B) suspended in 3% gelatin and injected into the right axilla at time 0. Five mice were killed at the specified times and cell suspensions prepared from the lymph nodes of the right and left axilla, spleen and the peripheral leukocytes collected. The cells were washed 3× in saline and then counted for residual radioactivity.

The distribution of labeled ELX in the tissues of recipients at varying times after administration was determined in three separate studies. The results of the first two are presented in tabular form (Table III). Although there were minor variations in the two experimental designs, the data agree substantially and, therefore, have been summed and averaged. The variations included subcutaneous vs. intraperitoneal route of injection, the use of two different preparations of ELX, and the inclusion of 0.5 ml of carrier ALS in one case. Control animals received ^{131}I labeled NRS IgG in 0.5 ml of whole NRS. The NRS IgG was found in highest concentration in the serum at 24 hr and disappeared from this compartment with a half-life of about 5.0 days. The distribution in the rest of the tissues was roughly related to the organ weight, i.e. liver > kidneys > lungs > femur > spleen. At all time intervals, the uptake of ELX in nonlymphoid organs was far less than that for normal rabbit IgG. At 24 hr, the uptake of ELX in lymphoid organs (lymph nodes, spleen , and thymus) exceeded that

of NRS IgG. Since the ratio between serum and tissue levels was so much lower in the ELX group, the observed counts cannot be simply a reflection of blood supply or tissue mass but must represent some specific localizing mechanism.

TABLE III

Percentage Distribution of ELX and NRS IgG in the Tissues with Time

Tissue	Day							
	1	2	3	4	6	8	10	12
Serum*	0.8 (12.1)‡	0.3 (9.1)	0.2	0.1 (7.5)	0.06	0.02 (3.8)	—	— (2.4)
Blood formed elements	0.2 (2.0)	0.02 (1.07)		0.01 (1.1)	—	— (0.5)	—	— (0.3)
Lymph nodes	0.3 (0.25)	0.12 (0.25)	0.04	0.03 (0.19)	0.01	— (0.09)	—	— (0.08)
Spleen	0.33 (0.16)	0.09 (0.18)	0.05	0.03 (0.17)	0.02	0.01 (0.08)	—	— (0.06)
Thymus	0.08 (0.06)	0.02 (0.10)	0.01	0.01 (0.09)	—	— (0.03)	—	— (0.02)
Liver	1.20 (2.30)	0.33 (3.50)	0.27	0.27 (1.70)	0.18	0.09 (0.80)	0.05	0.02 (0.70)
Kidneys	0.80 (1.10)	0.15 (1.50)	0.11	0.08 (0.82)		0.04 (0.40)		0.02 (0.32)
Lungs	0.26 (0.70)	0.07 (1.10)	0.04	0.03 (0.71)	0.01	— (0.30)	—	— (0.26)
Femur	0.13 (0.37)	0.05 (0.40)	0.06	0.02 (0.34)	0.02	0.01 (0.21)	—	— 0.12)

‡ Figures in parentheses represent distribution of NRS IgG. Numbers without parentheses are those found for ELX distribution. The results are the summed and averaged figures for two different experiments in which two different preparations were used (ELX I and II). Each figure represents the data from at least 4 individual animals.

* The values have been corrected for a serum volume of 0.5 ml.

Over the first 48 hr, the distribution of NRS IgG in the tissues is increasing, while at the same time the rate of disappearance of ELX is at a maximum. The elimination of ELX from lymphoid organs appears to take place more rapidly and more completely than that of nonlymphoid organs, e.g., liver, or kidney.

The uptake by lymphoid organs of specific antibody is at all times, from 24 hr on, extremely small.

Since the bulk of the ELX molecules are eliminated from the body during the first 24 hr, the third experiment examined the distribution of ELX at more frequent intervals during this period. Mice received 80 μg of labeled ELX intraperitoneally and were sacrificed at 2, 4, 8, 24, and 48 hr thereafter. The distribution of the label is depicted in Fig. 8. The peak uptake occurs at 4 hr and the

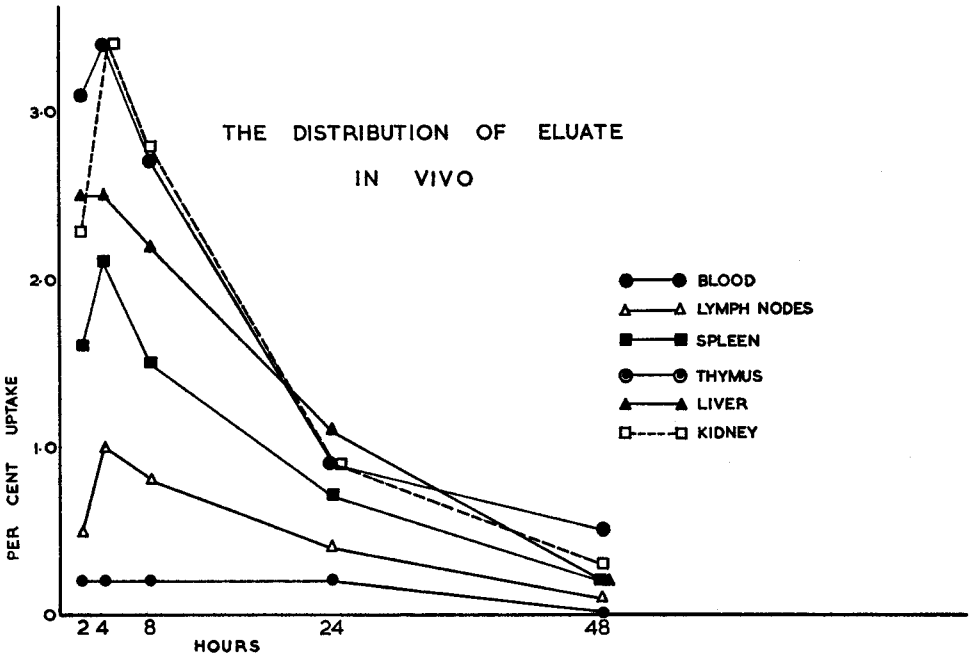

FIG. 8. Showing the percentage distribution of ^{131}I labeled ELX in the various tissues and organs of mice over the first 48 hr after intraperitoneal injection.

greatest concentration of antibody is found in the blood where almost 5% of the counts are recovered. The kidney, liver, and spleen contain in decreasing frequency another 8% between them, while 1% is recovered in the pooled inguinal, axillary, brachial, and mesenteric nodes, and 0.2% in the thymus. It is apparent that even by 4 hr a good percentage of the radioactivity has been cleared from the body and the rate of elimination from the tissues continues at a rapid pace until by 48 hr the summed percentage of the injected dose remaining in lymphoid organs is about 0.3%. The label has also been rapidly eliminated from nonlymphoid organs and only 0.6% is present in the serum.

Localization of ELX within Lymphoid Organs.—Two experiments were done

in which ELX was labeled with ^{125}I and injected into recipient mice which were killed at intervals and their tissues prepared for radioautography. The tissues were fixed in 10% formalin, sections cut from paraffin wax and mounted on slides subbed with chromate gelatin. The slides were dipped in Ilford L4 emulsion and exposed for 6–8 wk. After development the slides were stained with either methyl green pyronine or Congo red.

In the first experiment ^{125}I-labeled ELX was injected intraperitoneally and the animals were killed at 2, 4, 8, 24, and 48 hr. The lymph nodes (axillary and mesenteric), spleen, thymus, liver, and kidneys were removed and processed for radioautography as well as routine histological sections.

At 2 hr the heaviest concentration of label in lymph nodes was found on and about cells in the paracortical area. The true cortex including the germinal follicles was relatively spared and contained considerably less label (Fig. 9*a*, *b*). The label in the spleen was concentrated around the periphery of the Malphigian follicles (Fig. 10) whereas the thymic label was diffuse and light. In the kidney an extremely dense concentration of grains was found in the renal proximal tubules but no localization was seen in renal glomeruli or in the liver.

By 4 hr the intensity of label within lymph nodes had increased but the distribution was unchanged. The splenic label was now spread diffusely throughout the cells of the white pulp (Fig. 11) and the renal tubular label was now concentrated on the luminal surface of the cells (Fig. 12). The thymic cortex was somewhat more heavily labeled than the medulla but remained relatively light. Within the liver, label was clearly localized to the cytoplasm of Kupffer cells and was often associated with the phagocytosis of labeled leukocytes (Fig. 13*a*, *b*).

Over the next 40 hr there was a rapid and progressive decrease in the intensity of label without any change in the pattern of localization. From 8 hr on, the routine sections of lymph nodes showed depletion of small lymphocytes from the paracortical areas with evidence of local cell destruction, i.e. presence of pale "ghost" cells, tingible bodies, polymorphonuclear infiltrate, and nuclear debris within phagocytic cells (Fig. 14).

At 48 hr, only a very small amount of label remained in the lymphoid organs and was predominantly associated with phagocytes (Fig. 15).

In the second experiment 20 μg ^{125}I-labeled ELX was mixed with 3% gelatin and injected into the right axilla, subcutaneously. The object was to achieve a more localized and sustained exposure of the regional lymph nodes and to simulate our usual experimental protocol in which the administration of ALS is subcutaneous. The draining, contralateral nodes, spleen, liver, and smears of the buffy coat of the blood, were examined at 2, 6, and 24 hr postinjection. At 2 hr there was very heavy labeling within the marginal sinus and lymph and vascular channels of the draining node (Fig. 16). No significant amount of label was detected on buffy coat cells or within the spleen, liver, or contralateral nodes.

At 6 hr the label in the draining nodes was largely localized within the paracortical area with sparing of the true cortex as noted before. Some label could now be detected in the paracortical area (PCA) of the contralateral nodes and within Kupffer cells in the liver (Fig. 17). Approximately 10% of lymphocytes in the buffy coat of peripheral blood were heavily labeled (Fig. 18). Within the spleen, label on lymphoid cells was largely localized to the periphery of the lymphoid follicles. At 24 hr considerable cell death was seen in the PCA of the draining node (Fig. 19*a*, *b*), but not in either the contralateral nodes or the spleen. Label and cell debris was undergoing phagocytosis. The buffy coat cells were devoid of label and in the spleen the label was now diffusely spread over the white pulp.

DISCUSSION

Before any consideration can be given to findings based on the use of ELX, we must first be satisfied that the material recovered in the manner described possesses the same characteristics held by the active fraction in the starting material. Antibody purification by acid elution has been used by others to study the fate and action of specific antibody and has proved to be reliable in other models (11, 12). On the other hand, Woodruff (13) has also used acid elution to purify ALS and has tested his preparations in vitro. His product retained the ability to bind with, agglutinate, and stimulate lymphocytes but had greatly reduced cytotoxic properties. Horse globulin was used in his experiments and the acid elution was carried out at 37°C, whereas in our experiments rabbit globulin and elution in the cold were used. We do not know if these differences in protocol account for the difference in result.

The antibodies which may attach to the lymphocyte membrane preparation only represents the potentially active fraction. The net active fraction will be less, for surely some of these antibodies will be directed to antigens shared between lymphocytes and other tissues, and in vivo may well be absorbed preferentially elsewhere so that they never come to bear against lymphocytes. An improvement might be the absorption of ALS IgG with nonlymphoid tissue prior to enrichment. Some denaturation may be a necessary consequence of the treatment used, yet this would appear to be of minor magnitude. Acid-treated whole ALS retained in large parts its biological activity: acid-exposed NRS IgG was handled in recipients much like that of unadulterated IgG, and even prolonged acid exposure resulted in a gross denaturation of less than 20%. Moreover, the ELX preparation was a relatively homogeneous solution of IgG molecules and migrated on a sucrose gradient appropriately. It retained its capacity to bind specifically to lymphocytes antigens and most importantly was biologically highly active both in vitro and in vivo. Therefore, it seems reasonable to assume that the behavior of this preparation reflects the behavior of anti-lymphocyte antibodies in whole ALS.

These studies strongly suggest that the direct or primary action of a single dose of ALS must be quite evanescent however long its secondary biological effects may last. The bulk of injected material is eliminated from the body within 24 hr and over 90% is gone in 48 hr. The plot of disappearance curves from the whole body is closely paralleled by the elimination from the various organs and blood stream as well. Evidence from other sources points in the same direction. For instance both Pichlmayr et al. (14) and Taub and Ruszkiewicz (personal communication) have followed the disappearance of cytotoxic antibody from the blood stream after administration of a single dose. While the rate of appearance and decay is to some extent related to the route of injection, they have found that within a matter of hours cytotoxic antibody can no longer be detected.

When animals are intentionally preimmunized to ALS IgG then the elimination of a subsequent dose of IgG is extremely rapid, often being complete within 48 hr (15, 16). None the less, it has been shown that even under these circumstances ALS can exert a powerful immunosuppressive action (2, 4) although there is some diminution of effect.

The mechanism responsible for the very rapid clearance of active antibody eluate found in this study cannot be one of active immunization because, although ALS IgG is highly immunogenic (15, 16, 17, 18) the period of rapid clearance after injection into a virgin animal does not begin for about 6 days (15). Up to that point the disappearance curve parallels that of NRS IgG. Denatured material would be expected to be cleared rapidly but the controls included in this study make gross denaturation unlikely and at any rate this explanation would fail to account for the corroborative findings of other studies. The most likely explanation is that lymphocytes become coated with antibody and are either destroyed along with antibody or bring about antibody degradation and excretion some other way. Humphrey (19) has studied the clearance of immune complexes where antigen and antibody are individually labeled, and has found that both are simultaneously degraded. A question which arises is, can the rate of clearance be altered by saturating the system? In studies with Jooste (unpublished observations) ELX was given to weanling mice which had been in some cases exposed to a prior dose of ALS. The rate of clearance of ELX from the serum under these circumstances was more rapid than that of NRS IgG, but less rapid than in the studies reported above (half-life roughly 36 hr.). One possible interpretation is that the smaller number of available lymphocytes resulted in a diminished number of binding sites for passive clearance.

Although ELX molecules are found to localize within lymphoid tissue to a greater extent than would be expected if they did not possess an affinity for lymphoid antigens, the amount of antibody uptake involves only a minor fraction of the total dose (20). The combined uptake of ELX by all lymphoid organs

at peak concentration is less than that present in the blood at the same time. Considering that there are fewer lymphocytes in the blood than in lymphoid tissue it is clear that the ratio of antibody to cells is far greater in the blood than within lymphoid tissue. Furthermore, the antibody is cleared from lymphoid organs *pari passu* with the clearance from other organs and from the whole body. Although the antisera from which ELX was prepared were raised against thymocytes and thymocyte membrane was used in the absorption step, the specific uptake into the thymus remains quite low. When compared with nonlymphoid tissue (femur, lung) the uptake is comparable, but when compared with spleen or lymph node the uptake is far less. We construe this observation as additional indication that access of antibody to thymocytes in vivo is restricted. Hintz and Webber (21) and Denman and Frenkel (22) have studied the localization of ALS within the tissues of recipients using the entire IgG fraction and compared the uptake with that of NRS IgG. Their studies have the same inherent limitations as the similar studies we undertook before turning to the use of purified antibody (see above). Both authors found very little uptake of ALS IgG antibody molecules within lymphoid organs.

The radioautographic studies reported here suggest that there is an unequal partition of antibody within lymphoid tissue. The greatest concentration of label occurred within and around the vascular channels in the paracortical areas of the lymph nodes. These are precisely the areas which are subject to lymphoid depletion after ALS administration (23–26). In the spleen the label is first heaviest around cells in the periphery of the white pulp follicles but later is found rather diffusely throughout the follicle. The true cortex of lymph nodes was relatively spared although it is true that to some extent antibody was distributed throughout lymphoid tissue. Levey and Medawar (4) have also shown by fluorescent labeling techniques that ALS is able to coat lymphoid cells within organs in vivo. However, they did not determine to what extent the label represented nonantibody protein. The reason for this inequality in tissue localization is not entirely clear, however; at least three possibilities exist. The first presupposes that antibody selects sites of binding because the antigenic makeup of the cells varies within lymphoid tissue, and the cells in the paracortical area are more congenial. This explanation is unlikely since, other things being equal one would expect the highest concentration of label in the thymus which is clearly not the case. Another possibility is that the antibody molecules are found most heavily concentrated in those areas where the blood supply is the greatest. Cells lying close to the path of diffusion would have the opportunity to accumulate larger amounts of antibody than those lying at some distance. The difficulty with this explanation is, again, that the thymus is at least as vascular as lymph nodes; furthermore, it is hard to see how the change in splenic localization with time could be explained on this basis. A third possibility is that the areas in which the heaviest label was found lie in the pathway of migration of

cells in the recirculating pool (27). The supposition here is that the cells may have been heavily coated in the blood stream or lymph prior to entry into the lymphoid tissue.

The reason why cells in the thymus, lymph nodes, and spleen which are coated with antibody are not destroyed is also not entirely clear but a number of factors may come into play. Humphrey and Dourmashkin[2] have shown that for lysis of erythrocytes to be brought about by IgG antibody, about a thousand molecules must attach to the same cell. Woodruff et al. (3) have calculated that each lymphocyte can take up as many as 5.0×10^6 molecules on its surface but the exact number required for lysis is not known. Therefore, although lymphocytes in lymphoid tissue are exposed to IgG antibody the concentration may not be sufficient to cause irreversible damage. Another limiting factor might be the local availability of complement. As early as 1961, Waksman et al. (28) showed that after the administration of ALS there was a drop in the circulating complement levels. If, as is widely believed, complement is necessary for the direct immune destruction of cells (29, 30) then a limited availability of complement within lymphoid tissue might explain the relative protection these cells receive. A third possibility is that cells in the circulation when coated with antibody become opsonized and subject to phagocytosis by the cells of the RE system, (31, 32) but that within lymphoid tissue, coated lymphocytes are not exposed to these phagocytic cells to the same extent. Finally, one may draw the analogy to the rather similar phenomenon well known to those who have attempted to destroy tumor growths with antibody. While antibody may inhibit the growth of dissociated cells, they are ineffective or enhance the growth of solid tumor (33–35). At any rate, the exact explanation for this phenomenon will have to await further experimentation.

These findings cast grave doubts on the tenability of those hypotheses concerning the action of ALS which postulate the persistence of antibody molecules in the recipient. For instance, "blindfolding" (4, 36, 37), in which the lymphocytes are thought to be rendered incompetent because their receptor sites are covered by antibody, requires that an antibody coat persists throughout the period of incompetence. Similarly a peripheral enhancement mechanism (38) requires that significant amounts of antibody persist to coat graft antigen during the period of graft protection. In view of the rapid disappearance of antibody from the body and the very prolonged effects which may follow even a single dose of ALS these mechanisms become very improbable. These results do lend support to the evidence that ALS coats circulating lymphocytes and causes their removal from the circulation by the cells of the reticuloendothelial system, predominantly the liver (26). They also help to explain the relative protection enjoyed by cells within lymphoid tissue (39) and provide some explana-

[2] Humphrey, J. H., and R. R. Dourmashkin. 1968. Personal communication.

tion for the selective anatomical lesion caused within lymphoid organs (23-26). All in all they are in accord with the hypothesis that ALS achieves its immunosuppressive effect by bringing about a selective ablation of the population of recirculating lymphocytes (25).

These findings also bear upon the practical considerations of timing and dosage of ALS as applied to clinical use. For instance, it becomes clear that any schedule of administration in man will have to be predicated on a knowledge both of the turnover time of the recirculating pool of lymphocytes and of the effective rate of clearance of the specific active component of anti-human ALS.

SUMMARY

Studies designed to gain insight into the mechanism of action of the active component of antilymphocyte serum were carried out using an antibody eluate prepared from the IgG fraction of anti-lymphocyte serum by absorption and subsequent elution from thymocyte membrane. The resulting antibody eluate was labeled with radionuclide tracer to determine the fate of the antibody in vivo. The result indicated that anti-lymphocytic antibodies are eliminated from recipients extremely rapidly. The mechanism for this rapid clearance appears to depend upon the absorption of antibody molecules onto lymphocyte surfaces and the subsequent clearing and degradation of the antibody-lymphocyte complexes by the reticuloendothelial system.

Distribution studies confirm that the major site of antibody-lymphocyte interaction occurs in the periphery with relatively little penetration of antibody within lymphoid organs. Radioautographic studies showed that the pattern of localization within lymphoid and other organs is confined to rather specific areas. These observations are believed to offer strong support for the notion that anti-lymphocyte serum achieves its immunosuppressive effect by bringing about a selective ablation of the population of recirculating lymphocytes.

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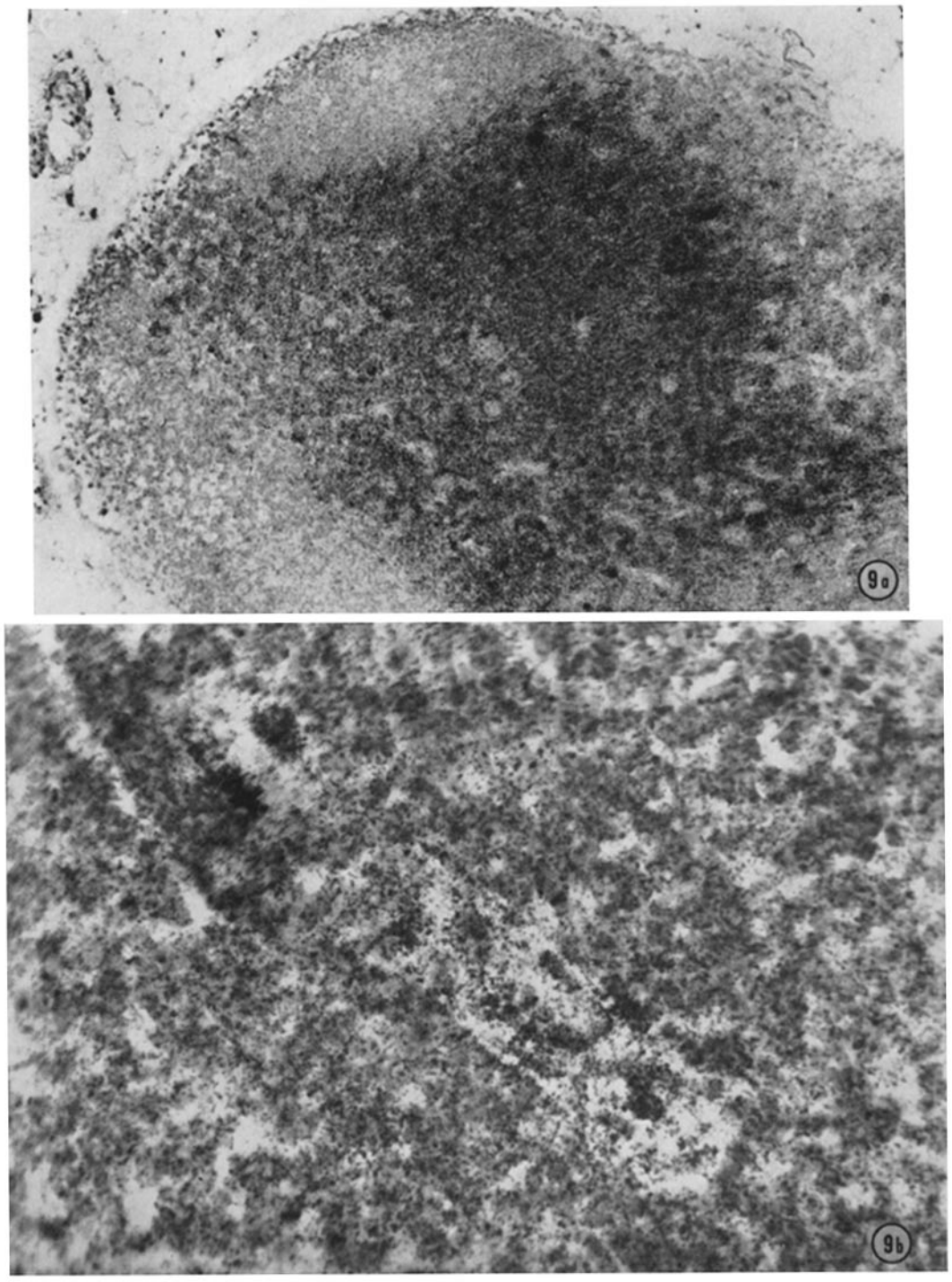
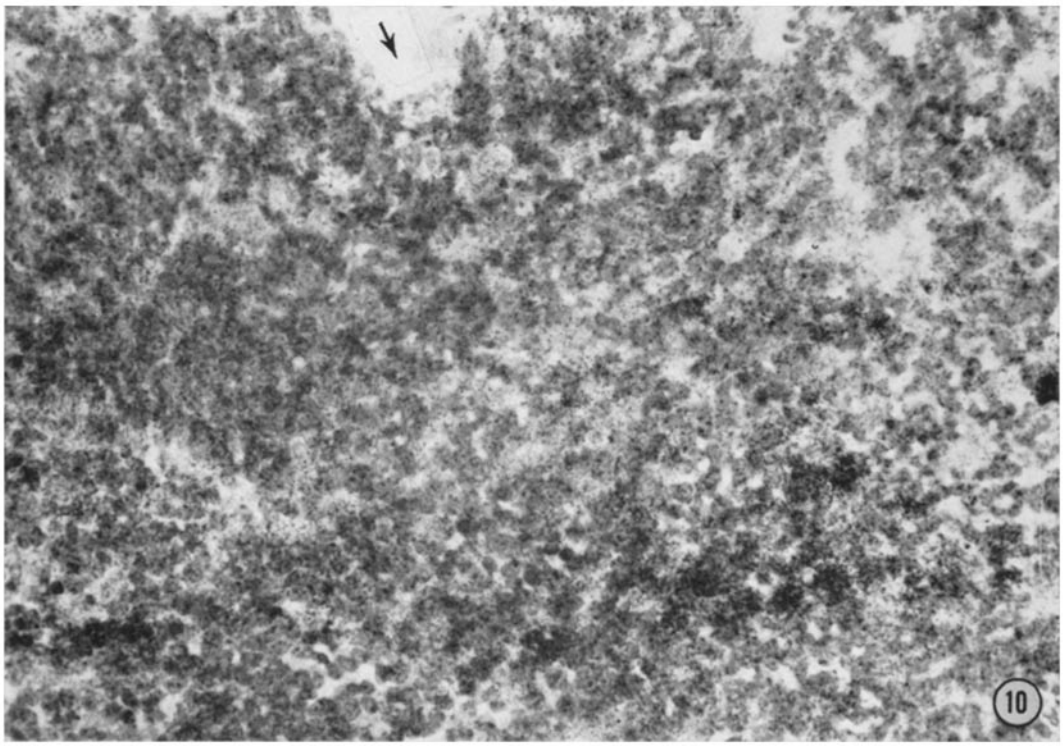


FIG. 9*a*. Axillary lymph node at 4 hr. Note the heavy label relatively circumscribed to the paracortical area with sparing of the cortex. (× 325)

FIG. 9*b*. Higher magnification of paracortical area showing labeling of all cells. Note the greater intensity of label within the vascular channel. (× 620)



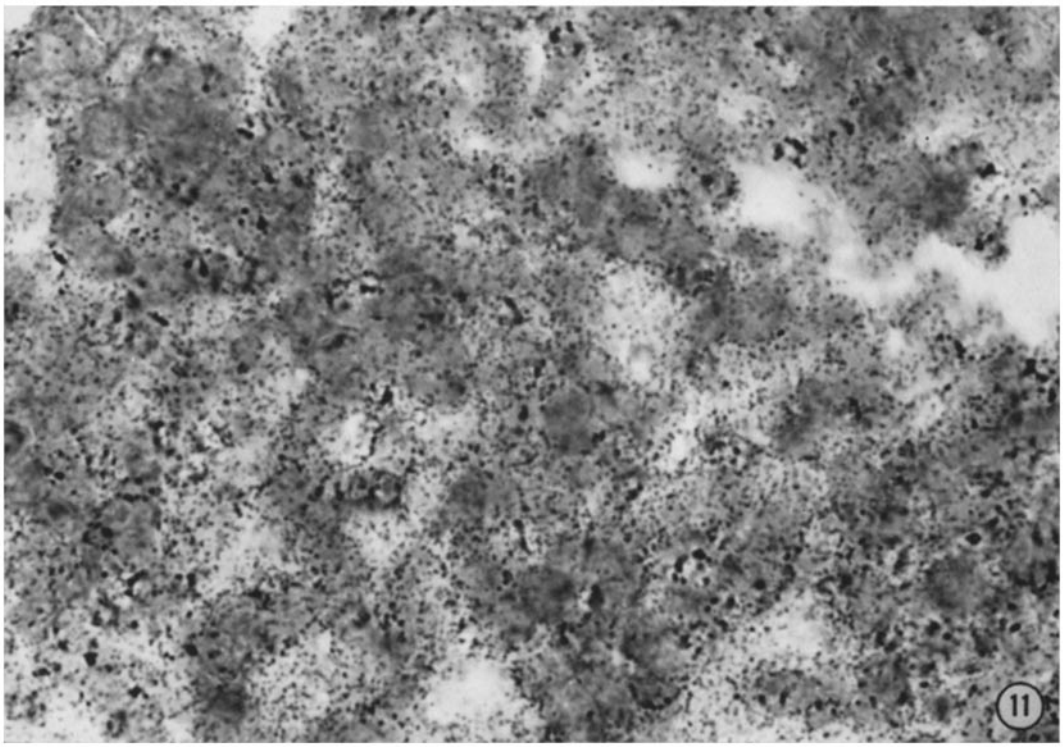
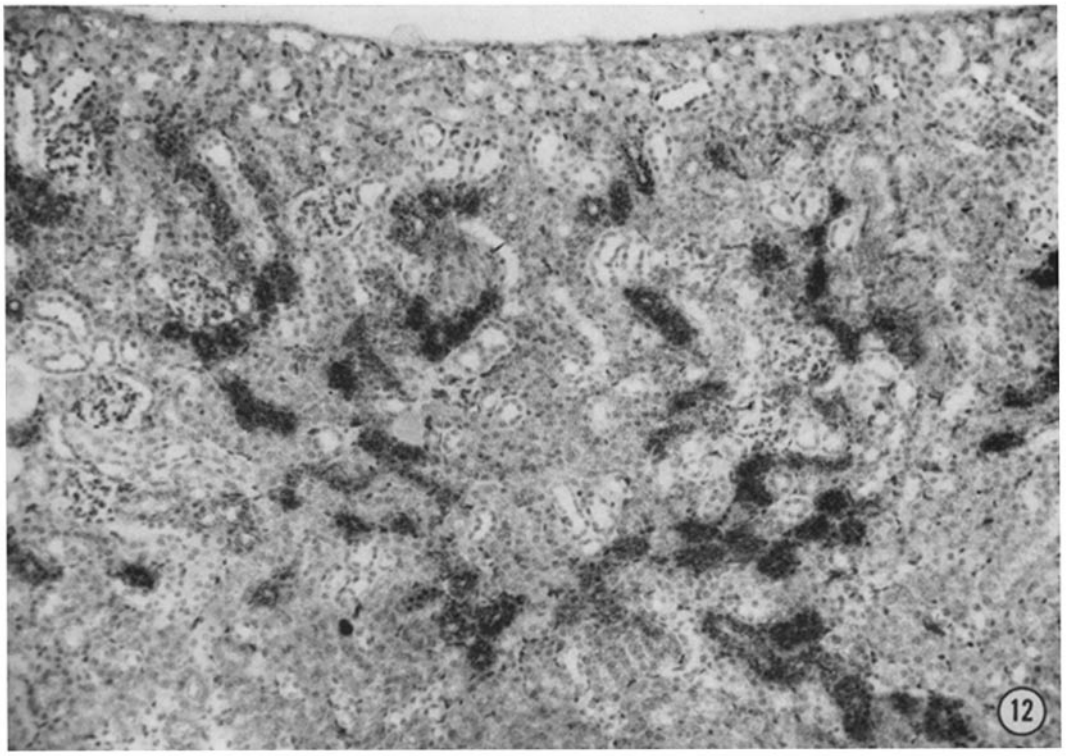


FIG. 10. Spleen at 2 hr. Arrow indicates a central arteriole. Note the heavy concentration of label associated with cells at the periphery of the Malphigian follicle. (× 365)

FIG. 11. Splenic white pulp at 4 hr. The label is diffusely spread over cells but clumps of label are already found in the cytoplasm of reticular cells. (× 740)



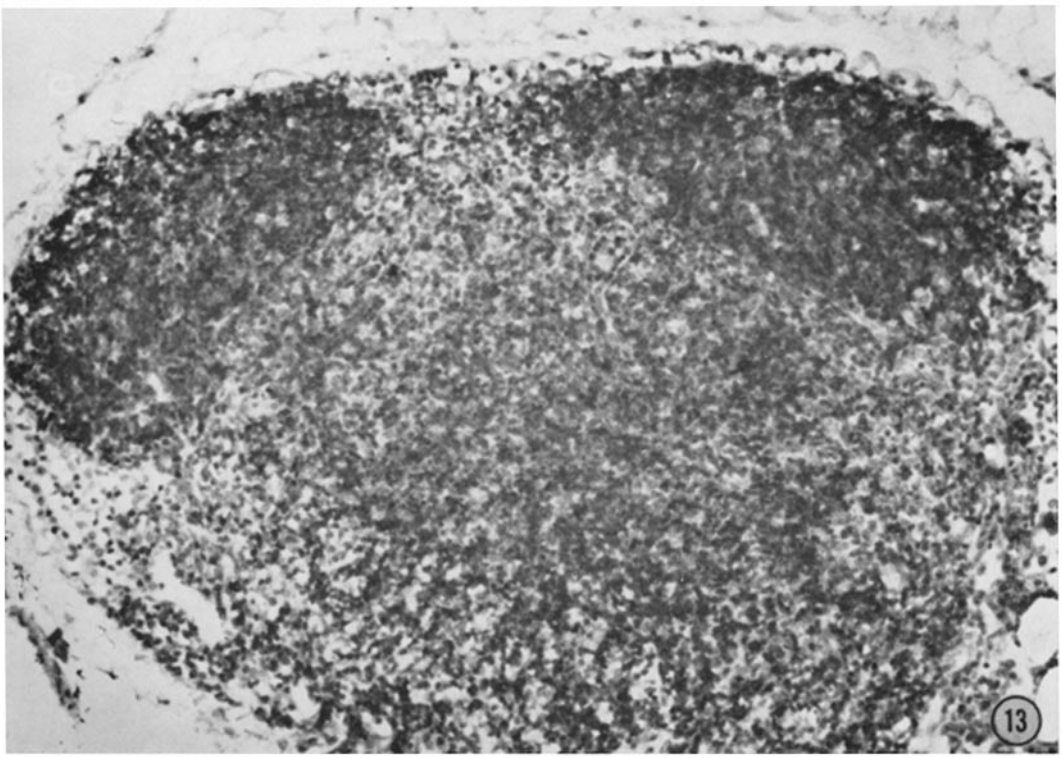


FIG. 12. Kidney at 4 hr. Note the very dense label in the proximal tubules and the sparing of glomeruli and other tubular components. (× 125)

FIG. 13. Routine section of an axillary lymph node 48 hr after ELX. Note the depletion of the PCA as contrasted with the cortex. (H and E × 210)

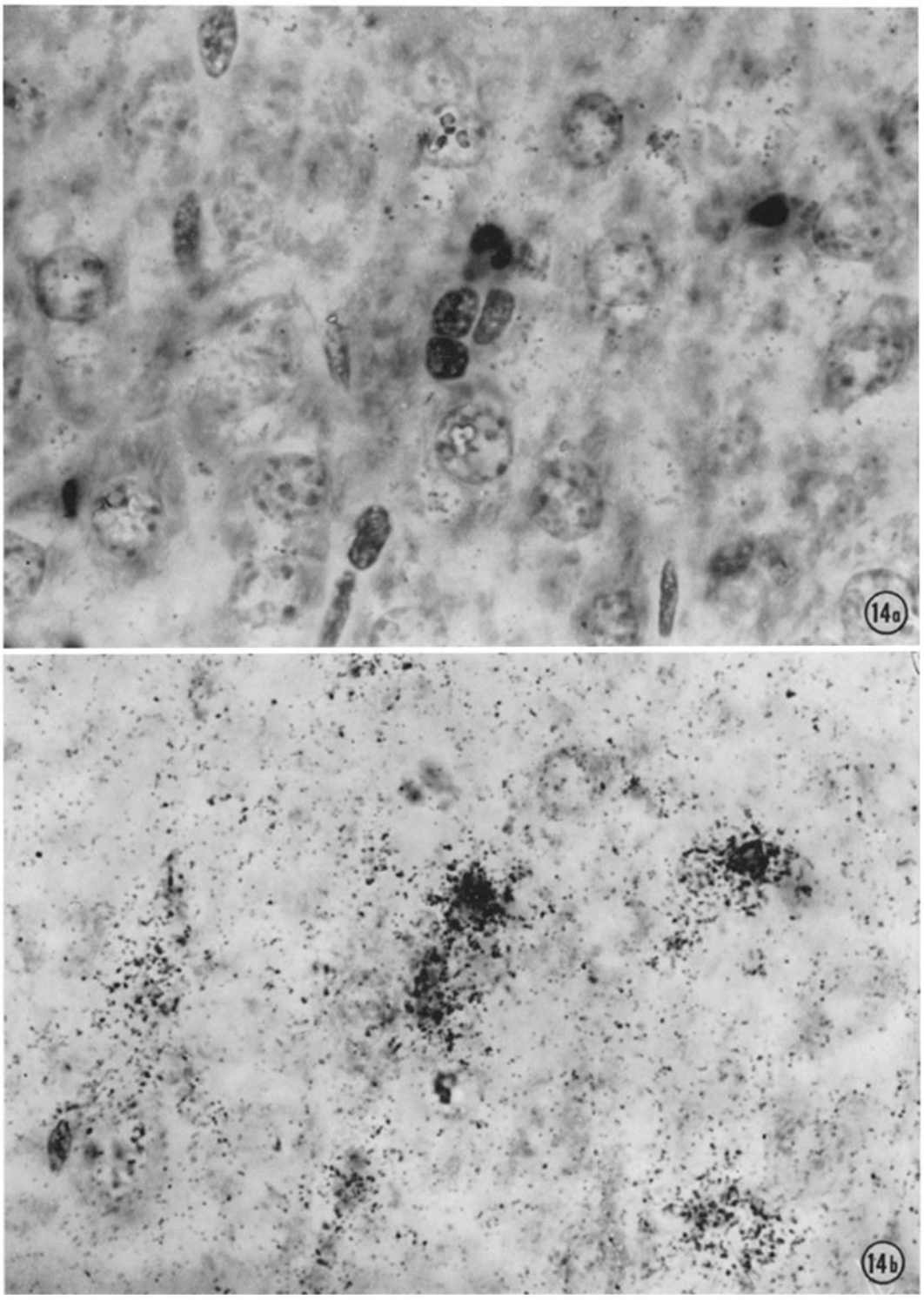
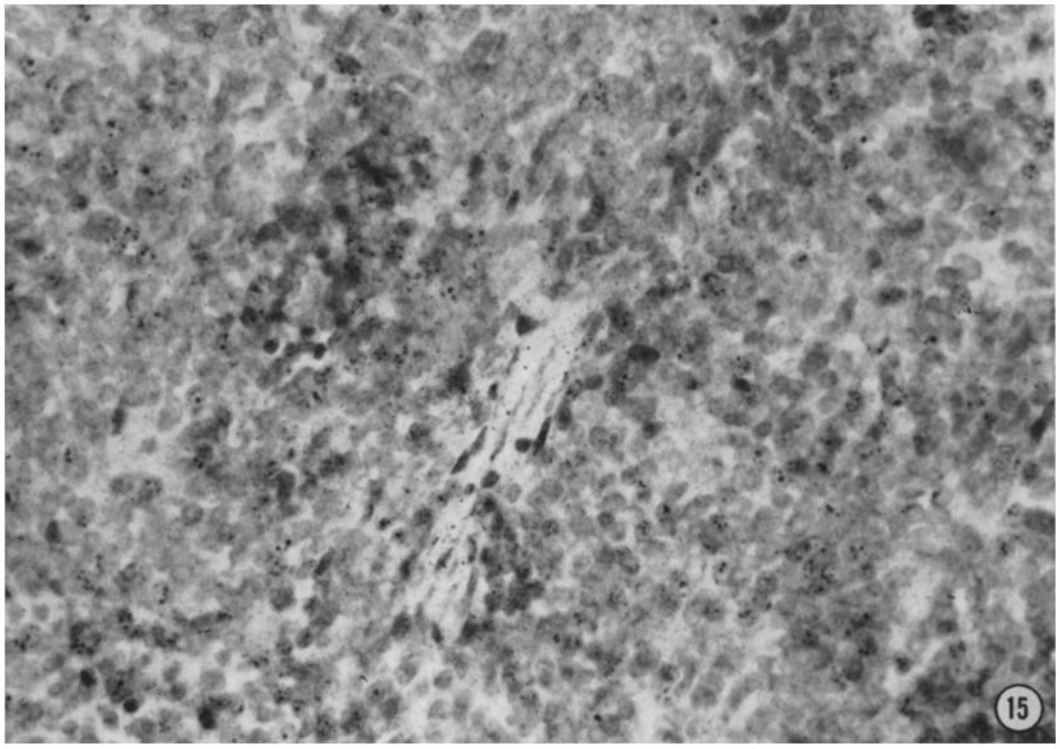


FIG. 14*a*, *b*. Two identical fields of the liver at 4 hr; *a* is focused on the cells and *b* on the grains. Within the Kupffer cells are phagocytosed leukocytes and in figure 14*b* it can be appreciated that it is these cells which are heavily labeled. (× 1200)



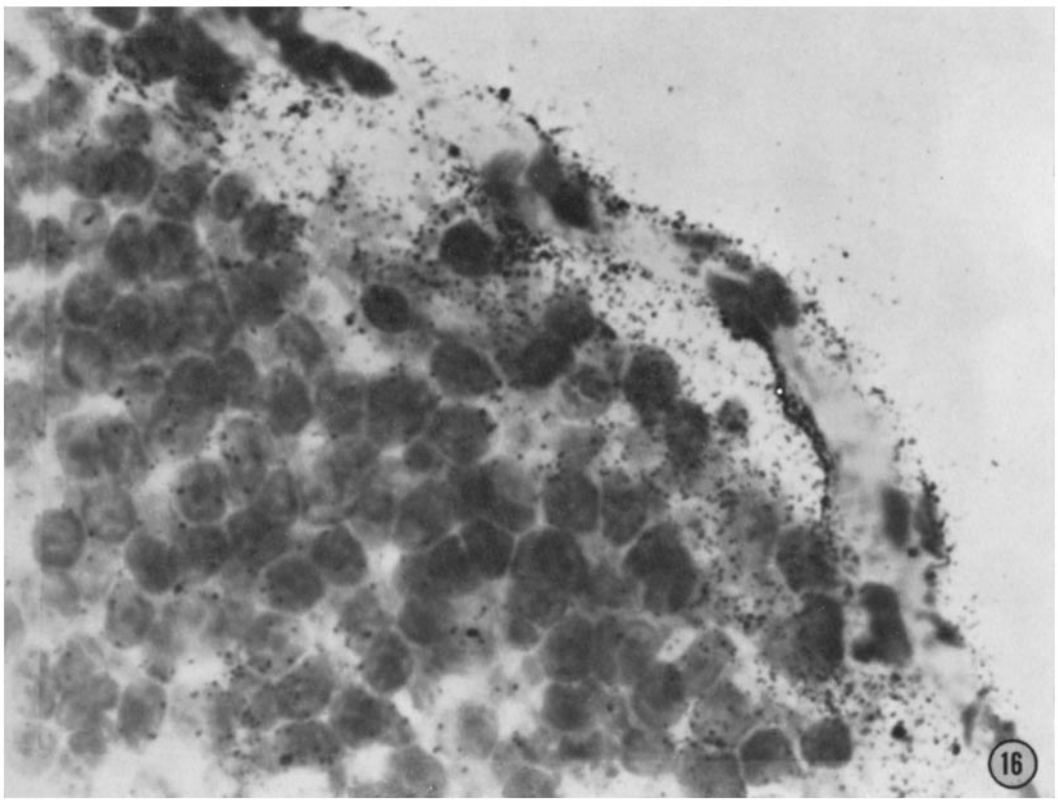


FIG. 15. Splenic white pulp at 48 hr. Note the marked decrease in label with that remaining associated with phagocytosis. (× 485)

FIG. 16. Draining lymph node 2 hr after subcutaneous injection of ELX ^{125}I. There is a heavy concentration of label in the marginal sinus. (× 1200)

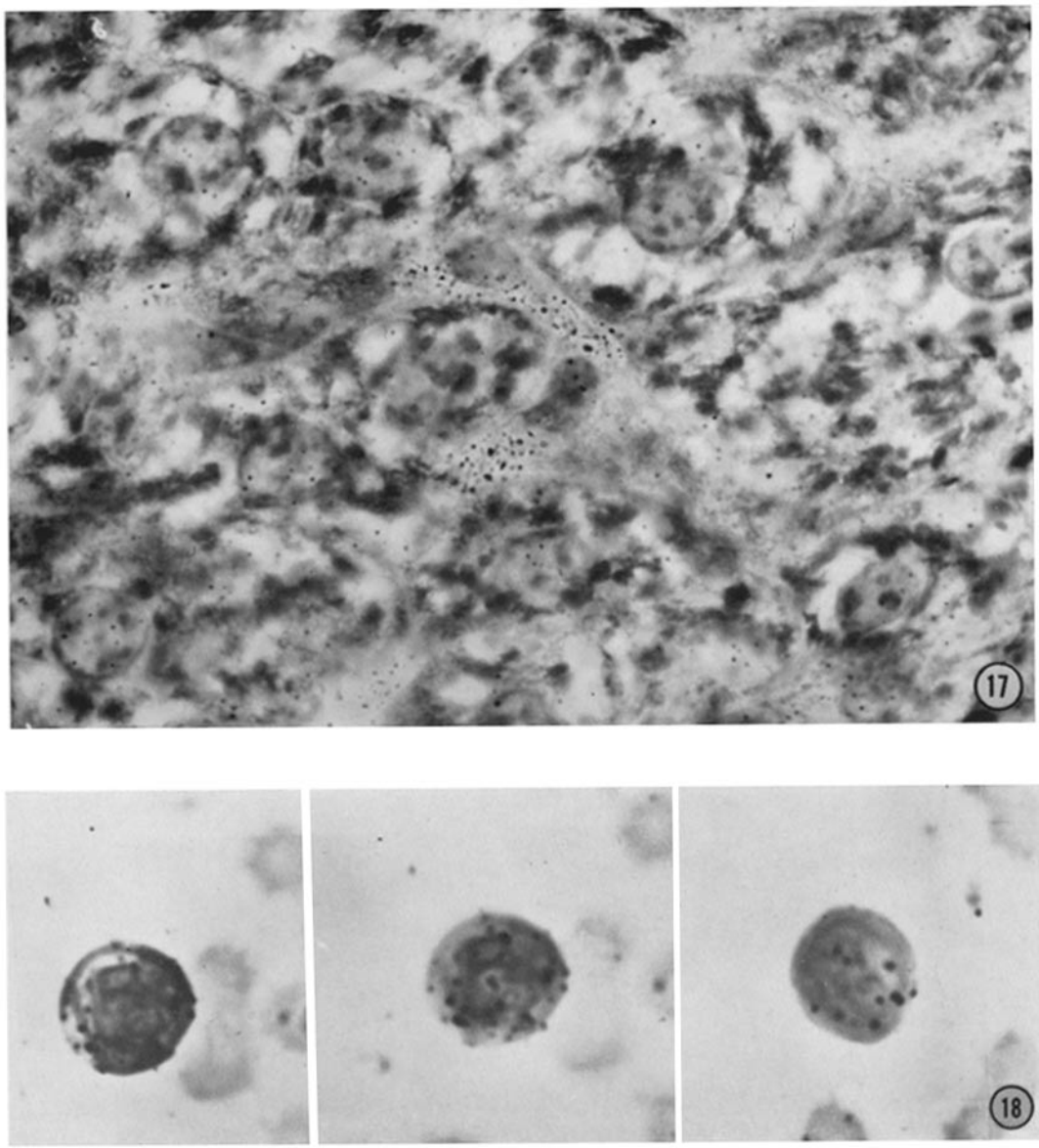


FIG. 17. Liver 6 hr after ELX ^{125}I subcutaneously. Note the specific localization of label within the cytoplasm of Kupffer cells. (× 1200)

FIG. 18. Labeled lymphocytes found in the peripheral blood smear at 6 hr. (× 2050)

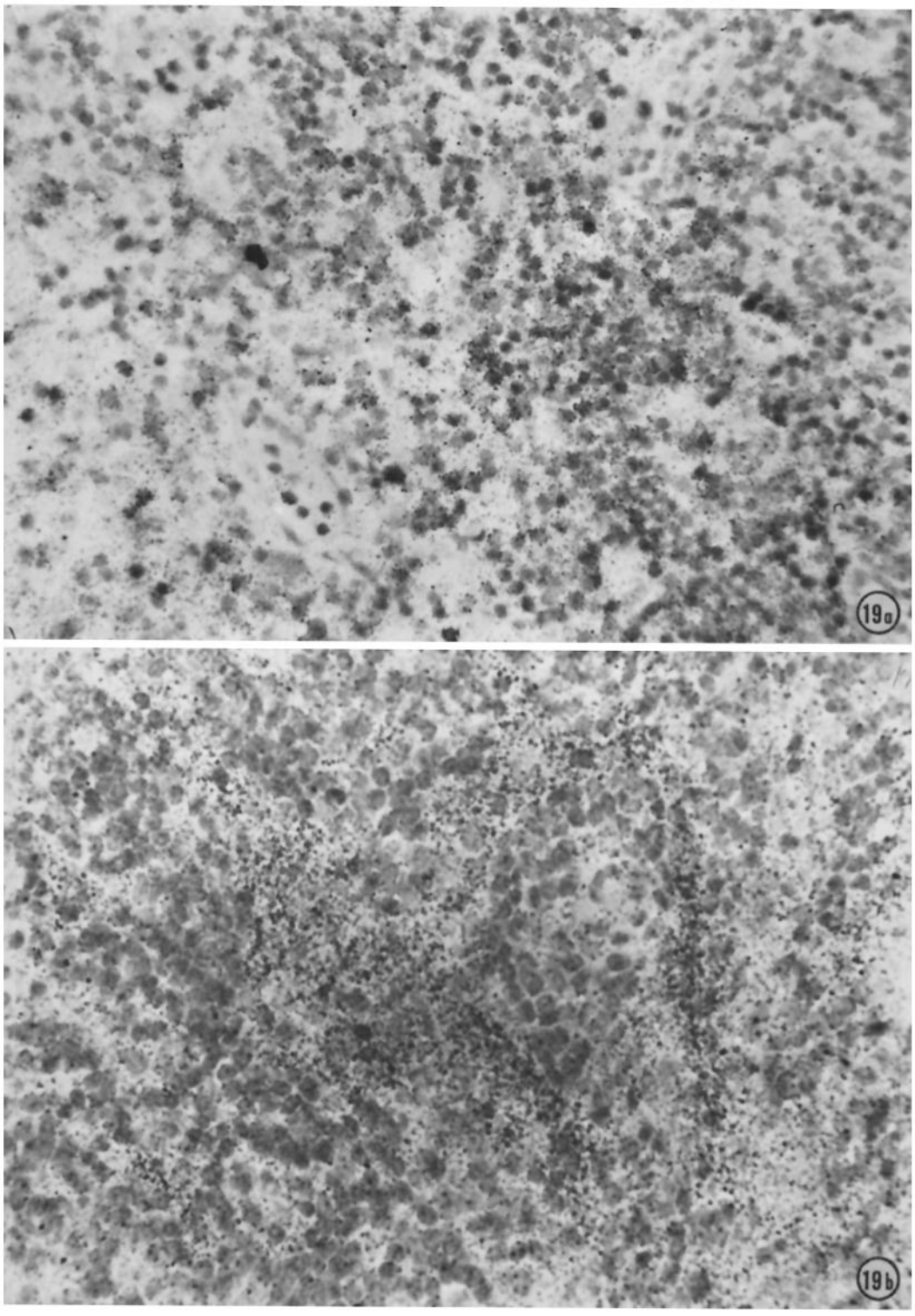


FIG. 19*a*. Paracortical area of an axillary lymph node at 24 hr. Lymphoid cell depletion is already evident and many pale necrobiotic cells are seen. The general intensity of labeling is decreasing. (× 485)

FIG. 19*b*. Axillary lymph node 24 hr after intraperitoneal injection of ^{125}I NRS IgG. Contrast the cellularity and localization of label with Fig. 19*a*. (× 485)