STUDIES WITH TRITIATED ENDOTOXIN

III. The Local Shwartzman Reaction

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The phenomenon of local tissue reactivity described by Shwartzman [1] consists of a lesion of the skin produced by the local injection of a gram-negative bacterial filtrate followed in an appropriate time interval by the intravenous injection of the same or similar material. The morphologic characteristics of the lesion and numerous variations in its production have been extensively described.[1-5] However, the mechanism by which this lesion develops has remained speculative. The proposed mechanisms of action of bacterial endotoxin in producing local lesions can be divided into 4 groups. These are: (a) direct action of the bacterial endotoxin on the vessel wall (muscle or endothelium) [6]; (b) stimulation of a hypersensitivity state of the delayed type [7]; (c) reaction of the endotoxin with cellular elements in the area and subsequent release of a vaso-active or toxic substance [8,9]; (d) alteration of the clotting mechanism with subsequent localized thrombosis [10] (provoking injection).

The use of a tagged endotoxin in conjunction with radioautographic localization in tissue may provide evidence in deciding the cellular sites of endotoxin distribution. This information should prove relevant in determining which of the above-listed mechanisms is valid. This study deals with our experience in the localization of a tritium-tagged *E. coli* endotoxin during production of the local Shwartzman reaction.

Material and Methods

One to 1.5 kg. albino rabbits were used throughout the experiment. They had free access to rabbit pellets, leafy vegetables and water.

As endotoxin, *E. coli* lipopolysaccharide O111:B_4 purchased from Difco Laboratories was used in all experiments. The endotoxin was exposed to 3 curies tritium gas for 10 days at 0° C. and 250 mm. Hg pressure according to the method of Wilzbach [11] (New England Nuclear Corporation, Boston, Mass.). Purification following tritiation was carried out as previously described [12] or by passing the tritiated

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material through a Sephadex G-100 column. Toxicity assays using the mouse lethality method previously described [12] were performed on each lot of purified material.

Group I. Local Injection of Tritiated Material (Preparative Injection)

Eighty albino rabbits were given intradermal injections of 250 μg. tritiated endotoxin into the posterior superior aspect of the right ear. Nontritiated, though otherwise similar, endotoxin (125 μg.) was injected into the left ear. All intradermal injections were made in 0.25 cc. of normal saline. In certain animals 200 μg. of the nontritiated endotoxin was introduced into the anterior left ear vein 24 hours after the intradermal injections. Additional animals were given injections as controls for variation in time interval between injections, and absence of local or intravenous injection. Animals were necropsied in groups of 4 at 0, 5, 15, 30 and 60 minutes, and 2, 4, 8, 16 and 24 hours after intradermal injection. Similar groups of 4 animals were sacrificed at 5, 15 and 60 minutes; 2, 6, 8, 24 and 48 hours, and 7 days after intravenous injection. Both ears, including the basal lymph nodes and adjacent drainage areas, were fixed in 10 per cent neutral formalin and a "Swiss roll" preparation made.[12] Paraffin sections were cut in quadruplicate at 6 μ, and 3 of these were placed under AR10 Kodak stripping film or NTB2 Kodak nuclear track emulsion. Exposure was interrupted at 5 days, 2 weeks, and 1 month and the radioautographs developed and stained as previously described.[12] The sections were numbered randomly and tabulated by 2 observers (W.S., B.W.) according to a predetermined outline. Using a 0 to 4+ grading system, characteristics of the lesion, the proximal drainage area, the perinodal region, and the lymph node were evaluated. Free tagged material, tagged cellular elements, tagged vascular elements and general morphologic alterations were considered.

Group II. Tritium Tagged Intravenous (Provocative) Dose

Intradermal injections (125 μg.) of nontritiated endotoxin were given into the left ear and 0.25 cc. saline in the right ear in 24 rabbits. Twenty-four hours later, 400 μg. tritiated endotoxin was injected into the anterior superior right ear vein. Animals were necropsied in pairs at 0, 30 and 60 minutes; 3, 6, 12, 24 and 48 hours and 1 week after intravenous injection. Radioautographs of paraffin sections of both ears, heart, lung, thymus, liver, spleen, adrenal, kidney, intestine and lymphoid tissue were made and exposed for 1 and 2 months. These were subsequently developed, stained and randomized for tabulation.

Group III. Method for Studies with Tritiated Zymosan

Purified Zymosan, reagent grade (Mann Research Laboratories, New York) was submitted for tritiation under the same conditions as the endotoxin specimens. Specific activity was determined as 55 μc. per mg. after purification by triple dialysis and centrifugation. The particles remained large and were suspended in saline with difficulty. Mouse lethality studies showed no deaths to levels of 2 mg. injected intraperitonally at increased ambient temperature.

Twelve albino rabbits received 250 μg. of tritiated Zymosan intradermally in the right ear and 250 μg. of nontritiated Zymosan in the left ear. Several of these were given an intravenous injection of 300 μg. of nontritiated endotoxin at 24 hours after the local Zymosan injection. Animals were necropsied in pairs at 0, 2, 12 and 24 hours after local injection and 2 and 12 hours after intravenous injection.

Eighteen animals were given intradermal injections of nontritiated Zymosan (250 μg.) into the left ear and either saline or nontritiated endotoxin into the right ear. Tritiated Zymosan (1,000 μg.) was injected via the right ear vein at 24 hours. Animals were necropsied in pairs at intervals to 1 week after the intravenous injection. Radioautographs of ears and other organs previously mentioned were prepared and evaluated.

Group IV. Method for Nitrogen Mustard Study

Thirty albino rabbits received intravenous injections of nitrogen mustard (Mustargen, Merck Sharp and Dohme, Philadelphia, Pa.) in a dosage of 1 mg. per pound body weight. Prior to and 4 days after this injection, total leukocyte and differential blood counts were obtained in each animal. Each animal then received tritiated endotoxin either as the preparing or provoking dose with nontritiated endotoxin as the alternative material. Pairs of animals were necropsied at appropriate time intervals and radioautographs of the tissues were examined.

Results

Characteristic gross lesions compatible with the local Shwartzman lesion were easily recognized in the rabbit's ear. These measured approximately 3 to 4 cm. in diameter and occasionally formed a tail-like extension toward the base of the ear. With the exception of 2 animals, central raised hemorrhage surrounded by edema appeared in all animals that had received the 2 properly spaced injections of either tritiated or nontritiated endotoxin. In these 2 exceptions, bilateral renal cortical necrosis indicative of the generalized Shwartzman reaction was found, and examination of the area of local injection showed only a minimal amount of tagged material or cellular reaction. We assumed the intradermal dose was accidentally injected into a vein. Results of systemic radioautographic studies in these animals will be reported in a later study.

With nitrogen mustard pretreatment or the use of Zymosan as either the preparing or provoking dose, no typical local gross lesions were evident. Edema and moderate erythema could be seen frequently; however, the later hemorrhage and necrosis did not appear. In the nitrogen mustard group given the intravenous provoking injection, lethality was 40 per cent and systemic abnormalities were evident. These consisted of diffuse pulmonary hemorrhage and marked hepatic congestion. Decreased lymphoid tissue was also apparent. Systemic abnormalities were not detected in any of the other experimental groups.

Microscopic Features

Group I. Tritiated Endotoxin as the Preparing Material. Microscopic localization of subcutaneously injected endotoxin could be divided into 4 phases. Phase one began immediately after injection and continued to 4 to 6 hours after injection. Free tagged material was rapidly picked up by the numerous large round and spindle-shaped cells in the dermis (Fig. 1). This appeared to be only a small part of the total radioactive material, however. Moderately large amounts of tagged material could be seen lining lymphoid channels, free and within endothelial cells of lymphatics (Fig. 2). Within the first 10 minutes, the draining lymph

nodes also showed significant amounts of tagged material in sinusoidal cells and adherent to sinusoidal walls (Fig. 3). Smaller amounts of tagged material could be seen in the proximal drainage area as well, and was assumed to have been carried there by lymphatics. At 4 hours, a large amount of tagged material still remained in the area of injection and proximal to it.

Phase two of this reaction involved infiltration by polymorphonuclear leukocytes (PMN's) in the area of injection and to a lesser degree proximal to it. Free tagged material remaining at this time was rapidly taken up by these cells which infiltrated in massive numbers (Fig. 4). By 12 to 16 hours after injection, PMN's became pyknotic, degranulated and began to lyse. This process continued through 24 hours after injection, when round cell infiltration (plasma cells, lymphocytes, histiocytes) was evident. PMN masses consisting of degenerate forms still retaining large amounts of tagged material could be easily found in the area of the lesion and especially in the proximal drainage areas. These plugs filled and obstructed large endothelial lined spaces which were most probably lymphatic channels (Fig. 5).

Evidence favoring this concept over the possibility that these were venous channels as has been frequently described is as follows:

1. The channels showed no muscular walls as larger veins often do.

2. The channels were numerous, very large, empty except for the PMN plugs, and they were continuous with perinodal sinuses. Veins in these preparations appeared less numerous, smaller, and were filled with red cells.

3. The PMN plugs within the channels contained much tagged material; this would be extremely unlikely were these structures veins.

4. Large molecular weight particles (India ink) injected intradermally at the tip of the ear 18 hours after the local injection of endotoxin appeared in the dilated channels within 5 minutes. The tagged PMN plugs differed markedly in appearance from the numerous venous thrombi evident shortly after intravenous injection of endotoxin. No tagged material was localized in either arteries or veins in the ear prior to intravenous injection.

Phase 3 of this reaction followed the intravenous injection of nontritiated endotoxin. Within 2 hours, there was a dilatation of venous channels followed quickly by the formation of venous thrombi. These were composed of red cells, platelets and a few nontagged leukocytes. As the thrombi became more numerous both in the region of the lesion and proximal to it, hemorrhage was evident in many areas (Fig. 6). At this time tagged material was still evident within clumps of degenerate leukocytes in the area; however, none was present within blood vessels.

Phase 4 consisted of repair of the lesion. By 24 hours after intra-

venous injection, large numbers of mononuclear cells had replaced the polymorphonuclear leukocytes. A few of these contained tagged debris. The majority of the tagged material, however, remained with the overlying necrotic crust of tissue. This eschar was sloughed by 1 week following extensive formation of granulation tissue beneath it. This, then, completed the local reaction. If no intravenous injection was given, the tagged endotoxin was retained in the area in moderately large amounts within large mononuclear cells. This slowly decreased over the next week. As yet, no longer term studies have been performed to determine the total duration of retention of tagged material after a single injection.

Careful examination of autoradiographs of liver, spleen, kidney, lung, lymphoid tissue, heart, thymus, adrenal and intestine showed no evidence of systemic spread of a locally injected tagged endotoxin. The autoradiographs were exposed for periods up to 2 months. No systemic excretion studies of urine or stool were carried out in this group.

Group II. H^3 Toxin as the Provoking Dose. Systemic localization of intravenous tagged endotoxin occurred within 5 minutes of injection. Since true quantitation was not possible in a study of this type, only estimates of the degree of tagging at various time intervals could be made. Liver, spleen and lung retained the greatest amount of tagged material throughout the experiment. Immediate heavy tag within Kupffer cells in the liver (Fig. 7), sinusoidal cells in the red pulp of the spleen (Fig. 8) and in the pulmonary capillaries (Fig. 9) was found. The localization to pulmonary capillaries was closely related to the very definite sequestration of labeled PMN's in these capillaries by 1 hour after injection. At 6 hours the number of tagged PMN's was markedly decreased within the pulmonary vasculature. Tagged material was then more evident in the capillary walls and large interstitial cells of the lung. By 24 hours the level of tagging in these 3 major organs began to decrease. Some tagged material, however, was still evident in the reticuloendothelial cells of the liver and spleen at the end of 1 week. At no time was there evidence of tagging in the hepatic cord cells of the liver or white pulp of the spleen. Numerous small vessels throughout the tissues showed patchy areas of tagging. These included glomerular capillaries, adrenal cortical sinusoids, myocardial vessels and small arteries and veins in the area of the ear lesion. There was no increased degree of tagging evident in the vessels of the local lesion as compared to other areas of skin.

Examination of lymph nodes, thymus, pancreas and intestine in each of the groups revealed no localization of tagged material at any time period. Evaluation of several animals given only a single large intravenous injection of tagged endotoxin [18] showed no recognizable difference in tissue distribution from that described above.

Group III. Results of the Use of Tritiated Zymosan as a Control Material. Use of tagged Zymosan as either the preparing or provoking dose resulted in no gross evidence of lesion production. Radioautographs following local injection of tagged Zymosan revealed larger masses of silver grains suggesting that the Zymosan particles were much larger than the particles of tagged endotoxin. These large particles remained free or were taken up by the large fixed phagocytes of the area. Lymphatic channels drained much of the material away from the area of injection within the first 2 hours. PMN infiltration began after 2 hours and was marked by 6 hours. However, tagged Zymosan was not taken up by PMN's to any significant degree (Fig. 10). The PMN's slowly left the area by 18 to 24 hours and were replaced by mononuclear cells. The marked degeneration of the PMN's evident with tritiated endotoxin was not found following local Zymosan injection.

Systemic localization of tagged Zymosan was identical to that following systemic endotoxin injection. Moderate sequestration of PMN's in the lung capillaries occurred within an hour after injection and occasional clusters of PMN's surrounded the large Zymosan particles. Deposition of Zymosan within the liver appeared much less intense than the deposition of endotoxin, but followed a similar morphologic distribution (Fig. 11).

Group IV. Results After Pretreatment with Nitrogen Mustard. Radioautographic distribution of locally injected tagged endotoxin was identical to that seen in group I during the first 4 hours after injection. Large mononuclear cells and lymphatics avidly picked up the material, and moderate amounts were evident in the draining lymph node. Blood vessels showed no evidence of endotoxin localization. PMN's were absent at all time intervals (Fig. 12). Drainage of the tagged material from the ear appeared to be increased, and by 24 hours only a small amount of radioactivity remained in the ear and lymph node. This suggested that the tagged endotoxin might have been emptied into the circulation by the lymphatic system. Only very minimal systemic localization of this material in the reticuloendothelial elements of the liver, spleen and occasionally in the lung was found after local injection, however.

No alteration in the histologic pattern of the local lesion area was evident after intravenous injection of tagged endotoxin. Distribution of this material in nitrogen mustard-pretreated animals was identical to that described previously[13] except for the definite decrease in tagged PMN's in the lungs and spleen.

Discussion

Previous attempts to localize injected endotoxins by fluorescent antibody techniques[14,15] and high energy beta emitting radioisotope tags

have provided some information regarding the fate of this material in experimental animals.[16] The use of tritium-labeled material and radioautography in this study has allowed a direct, specific localization of tagged substances at a cellular level. From these experiments, the proposed indirect action of endotoxin in the production of tissue lesions gains added support.

Studies of the local toxic effects of injected antigenic materials have incriminated the polymorphonuclear leukocyte (PMN) as an intermediate in these reactions.[8,17] The massive uptake of tagged endotoxin by PMN's as well as the rapidity of autolytic change in these cells following endotoxin uptake adds support to previous theories of PMN involvement. The localization of tritium-tagged Zymosan, a nontoxic, large, polysaccharide particle which is similar to endotoxin in several aspects [18] showed a minimal uptake by PMN's and a similar lack of increased autolytic change of the PMN with time. The corresponding lack of vascular thrombosis and necrosis when substituting tagged Zymosan as the preparing injection of the local Shwartzman reaction suggested that participation of PMN's was necessary for development of toxic vascular changes. Despite the retention of significant amounts of tagged material in the area of injection, suppression of PMN's by nitrogen mustard appeared directly related to the absence of lesion formation. The necessity of at least an 8 to 12 hour interval between preparing and provoking injections in the local Shwartzman reaction [1] could also be correlated with the time interval between primary injection and maximum PMN infiltration.

Other cellular elements were markedly involved in uptake of tagged endotoxin and also require consideration. Immediate and marked uptake by fixed phagocytes and by lymphatic endothelium was evident within 5 minutes after local injection. However, these cells were similarly involved after preparation by nitrogen mustard and after tritiated Zymosan injection. This would make implication of these substances more difficult in this reaction. The possibility that the cells produced vaso-active substances such as histamine after exposure to endotoxin has been suggested by the work of Schayer.[19] By using the experimental conditions referred to in this study, variation of tissue histamine in the area of local injection with time is now being investigated in this laboratory.

The lack of involvement of blood vessels after local injection of tagged endotoxin merits discussion. Direct vascular damage has been related by other investigators to vessel localization of antigen-antibody complexes in hypersensitivity states such as the Arthus phenomenon.[20] If a so-called "natural immunization" to endotoxins is present in normal rabbits as has been postulated,[7] a similar mechanism to that operating in

the Arthus reaction could be operative in the local Shwartzman reaction. Our studies indicated, however, that there was no significant direct localization of tagged endotoxin in either arteries or veins after intradermal injection and no increased localization here after intravenous injection of tagged endotoxin. The disparity between the degree of systemic vascular localization by radioautography following tritiated endotoxin injection and that reported following endotoxin localization of a fluorescein-tagged endotoxin by fluorescent antibody technique [15] becomes important when considering possible direct vascular action of endotoxins. Three explanations for this difference are possible. First, the use of a lethal dose in the latter study represents a 10-fold increase over that amount injected systemically in our study. Thus it may be that the vascular localization described by Rubenstein, Fine and Coons [15] represented an "overflow" phenomenon after saturation of the reticuloendothelial system. A second possibility is that the tag used either in our study or in the fluorescent study is not representative of the toxic portion of bacterial endotoxin (lipopolysaccharide). Since the fluorescent studies measure an indirect tag and antibody is produced to the entire "O" antigen, there is a distinct possibility of labeling the inactive protein portion of endotoxin which is present in almost all commercially prepared endotoxins. Such a possibility appeared remote when one used a direct method of observation such as radioautography and worked with a material that was diffusely tagged as has been previously shown for tritiated endotoxin.[12] A third alternative is the sensitivity difference in the two techniques. Our experiments indicated that the direct effect of endotoxin upon vessels in the local Shwartzman reaction appeared to be unlikely. The possibility that very minute amounts of endotoxin would be revealed in blood vessel walls by fluorescent antibody methods must be considered, however.

The role played by lymphatics in disseminating endotoxin in the adjacent tissue as well as removing it from the local site of action was evident in this study. Immediate drainage of free tagged material and the later removal of degenerate leukocytic elements might have been of primary importance in the degree of damage which resulted from local injection. Obstruction of this escape mechanism by PMN plugs late in the second stage of the lesion could be significant in the progression of the lesion. The significance of this finding depends on the degree of activity of injected endotoxin and the mechanism by which the venous thrombosis occurred. If this thrombosis depended on an alteration in the clotting mechanism, as has been suggested,[10] continued damage to venous endothelium by toxic products prior to the thrombosis would appear necessary. Since the degree of toxicity of residual endotoxin at long

intervals after injection is unknown, the effect of its retention in the lesion area must remain speculative.

Summary

A tritium-labeled bacterial endotoxin has been localized in rabbit tissue sections by radioautography during production of the local Shwartzman reaction. It is concluded from this study that: (1) Polymorphonuclear leukocytes (PMN's) picked up large quantities of endotoxin and underwent rapid autolytic change. Lack of PMN phagocytosis of endotoxin resulted in absence of lesion formation. (2) Blood vessels in the lesion area did not appear directly involved in endotoxin localization. (3) Lymphatics removed moderate amounts of tagged endotoxin but became obstructed by leukocyte plugs late in the second stage of the lesion. Much of the locally injected endotoxin or its breakdown products remained in the area of injection. (4) Intravenously administered tagged endotoxin was rapidly taken up by the reticuloendothelial system and to a much lesser degree by small blood vessels.

These findings support the proposed indirect action of bacterial endotoxin upon blood vessels in the production of the local Schwartzman reaction.

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LEGENDS FOR FIGURES

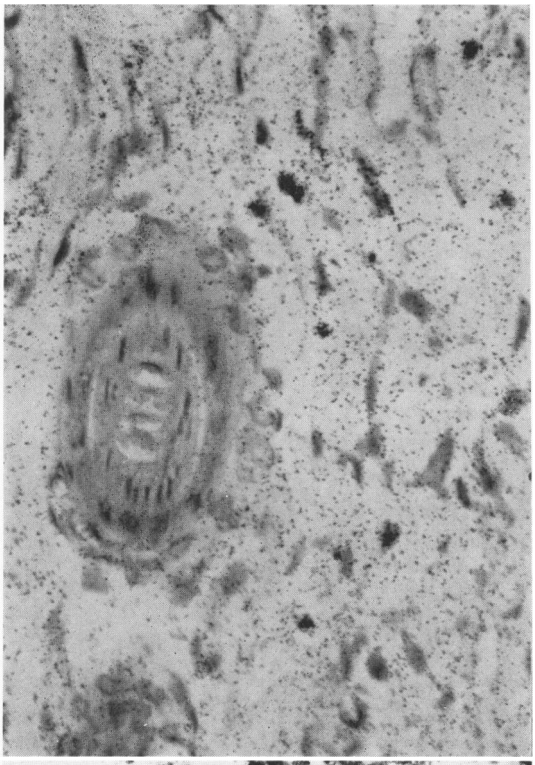
Photographs are of radioautographs prepared from tissue sections and stained with hematoxylin and eosin.

FIG. 1. A rabbit ear 5 minutes after intradermal injection of 250 μg. of tagged endotoxin. Round and spindle-shaped cells are heavily tagged, and there is also a heavy free tag in the area. × 250.

FIG. 2. Draining lymph channels 15 minutes after intradermal injection of 250 μg. of tritiated endotoxin. × 100.

FIG. 3. A draining lymph node 30 minutes after the intradermal injection of 250 μg. tritiated endotoxin. × 480.

FIG. 4. Dermal reaction in a rabbit ear 16 hours after the local injection of labeled endotoxin. Numerous tagged leukocytes are undergoing degeneration. Large black masses represent tagged material retained by fixed phagocytic cells as in Figure 1. × 250.



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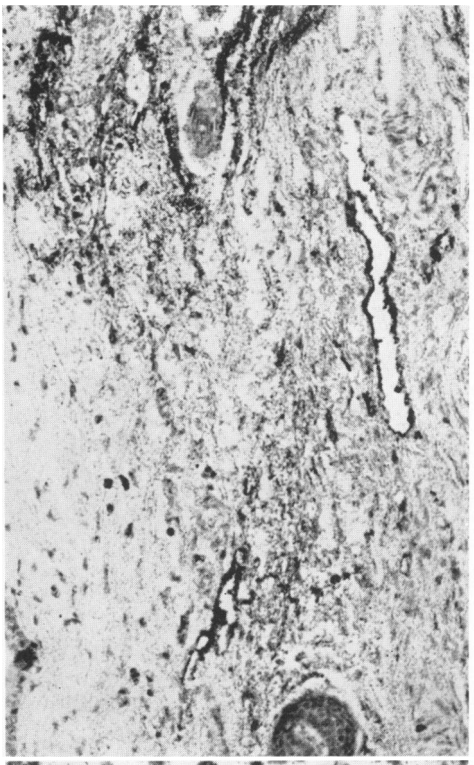

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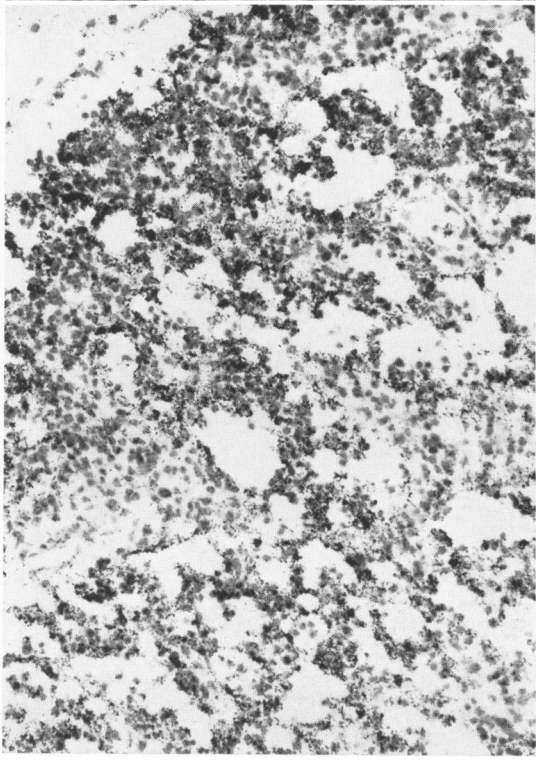

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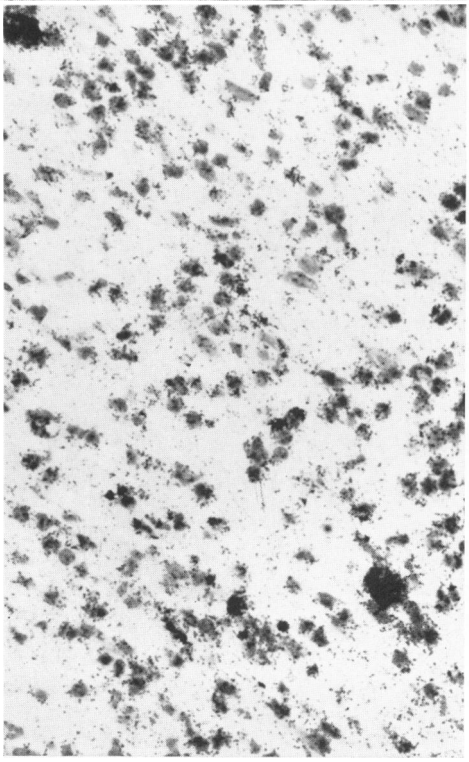

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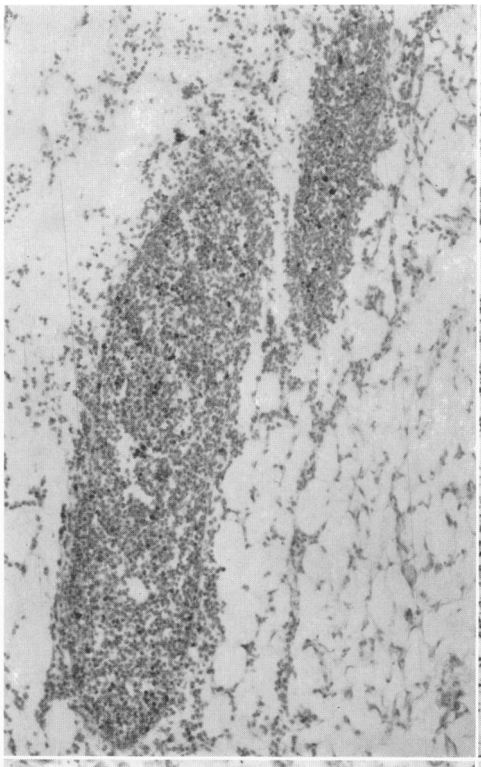
FIG. 5. A rabbit ear showing lymphoid spaces filled by masses of tagged PMN's at 24 hours after the local injection of tagged endotoxin. × 100.

FIG. 6. A rabbit ear 6 hours after the intravenous provoking injection of 200 μg. of nontagged endotoxin and 30 hours after the local injection of 250 μg. of tritiated endotoxin. A thrombosed vein near the bottom of the photo is surrounded by hemorrhage. × 100.

FIG. 7. Liver 1 hour after an intravenous provoking injection of 400 μg. tagged endotoxin and 25 hours after local injection of nontagged endotoxin. × 250.

FIG. 8. Spleen. Masses of silver grains appear over sinusoidal cells of the red pulp 1 hour after the intravenous provoking injection of tagged endotoxin and 25 hours after the intradermal injection of nontagged endotoxin. × 480.



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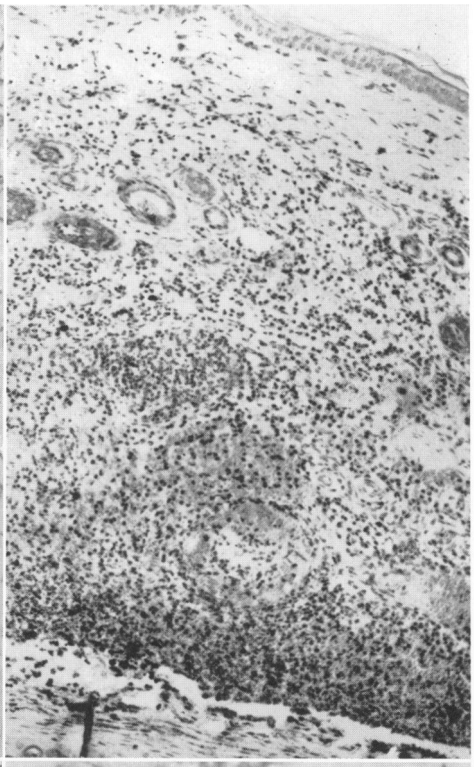

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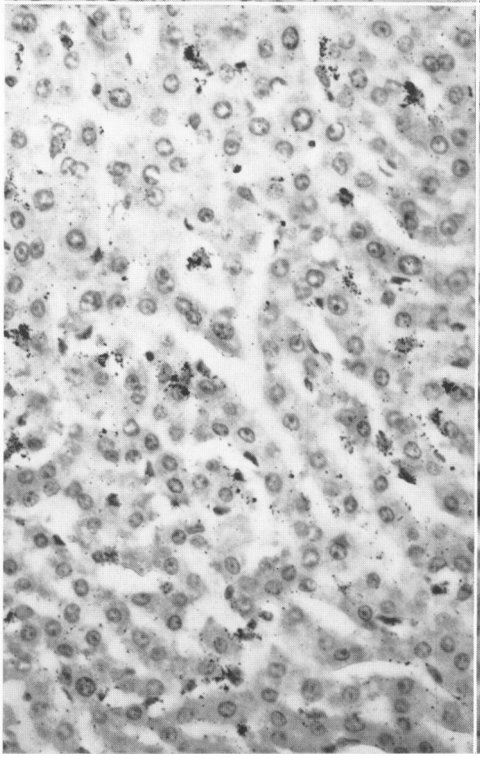

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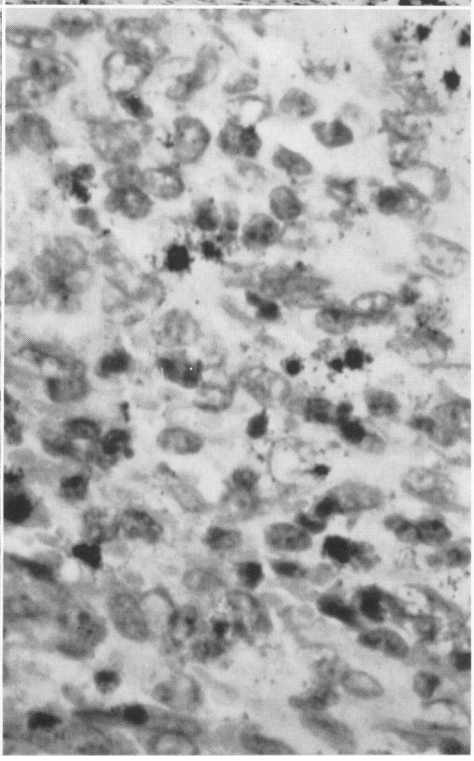

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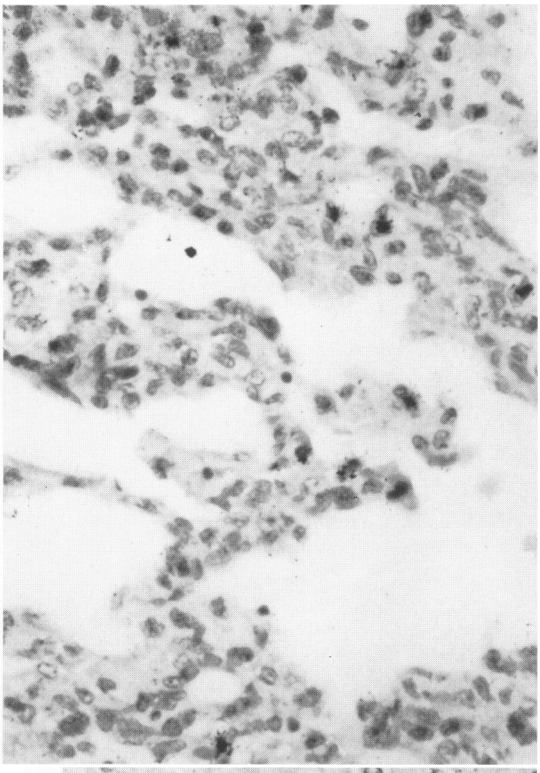
FIG. 9. Lung from a rabbit 1 hour after the intravenous provoking injection of tagged endotoxin. Tagged material is seen in both PMN's and pulmonary interstitial cells. × 250.

FIG. 10. A rabbit ear 24 hours after the local injection of 250 μg. of tagged Zymosan. Heavy tagging is evident over mononuclear cells in the area; a minimal degree of tagged material appears within PMN's. × 250.

FIG. 11. Liver from a rabbit given 1,000 μg. tagged Zymosan intravenously 1 hour earlier and intradermal nontagged endotoxin 25 hours earlier. The diffuse fine graining represents technical artifact. × 250.

FIG. 12. A rabbit ear showing heavy tagging of fixed mononuclear cells 12 hours after the intradermal injection of tagged endotoxin in a rabbit pretreated with nitrogen mustard. × 480.



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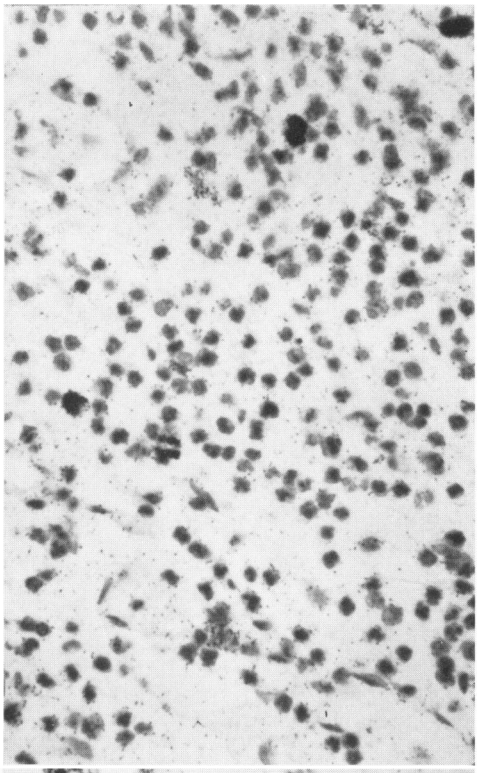

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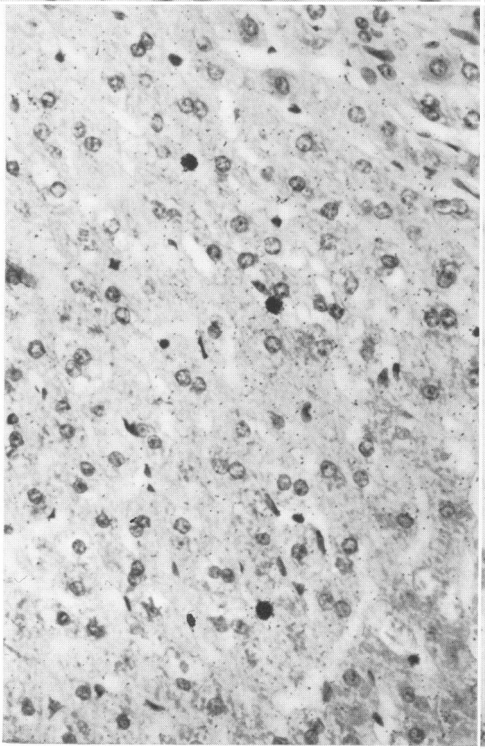

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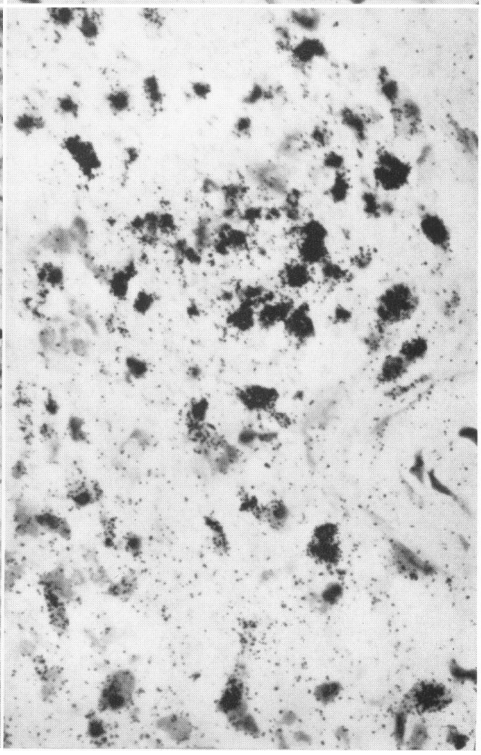

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