The Production and Passive Transfer of Allergic Adrenalitis

Seymour Levine, M.D., and Eugene J. Wenk, M.A.

THERE HAVE BEEN few experimental studies of allergic adrenalitis, although autoimmune injury to the adrenal gland may be the cause of idiopathic Addison's disease in humans.[1,2] Adrenalitis has been described only in guinea pigs and rabbits.[3–7] Its immunologic nature has been deduced from the presence of humoral antibodies and by analogy with better studied conditions such as experimental allergic encephalomyelitis (EAE).[8–12] However, adrenalitis has not heretofore been transferred passively with cells or serum. The present work concerns the production of adrenalitis in the rat, by active immunization with adrenal antigen, and by passive transfer with living lymphoid cells.

Methods

The experimental animals were male 8–12-week-old Lewis rats, except as otherwise specified. They were obtained from Microbiological Associates, Inc., and were maintained on Purina Laboratory Chow and tap water. Adrenal tissue was obtained from guinea pigs (heterologous); Wistar-Furth, LxBN, and CDF rats (homologous); and Lewis rats (isologous). The adrenals were dissected free of fat, rinsed in saline, and stored frozen. On the day of injection, they were thawed, chopped with scalpels, strained through a metal sieve, suspended in sterile saline, and homogenized by cycling between 2 syringes connected with a 20-gauge double-hubbed needle. The preparation was not heated and no preservatives were added.

Adrenal homogenate was injected with Freund's adjuvant, pertussis vaccine, both, or neither, according to one of the following methods:

1. Forty per cent (w/v) adrenal homogenate was emulsified in an equal volume of Freund's complete adjuvant (8.5 parts Bayol F mineral oil, 1.5 parts Arlacel A emulsifying agent, 4 mg./ml. of killed tubercle bacilli. The 0.05 ml. of homogenate injected intradermally into one of the right hind foot pads contained 10 mg. (wet weight) of adrenal tissue.

2. Same as above, but 0.05 ml. of concentrated pertussis vaccine (10 billion organisms) was injected intradermally into the dorsum of the same foot immediately thereafter.

3 and 4. Same as Methods 1 and 2 except that Freund's adjuvant was "incomplete" (lacked tubercle bacilli).

5. Five parts 80% adrenal homogenate were mixed with 1 part concentrated pertussis vaccine, and 0.30 ml. of the mixture, containing 200 mg. adrenal tissue and

From the Department of Pathology, New York Medical College Center for Chronic Disease, Bird S. Coler Hospital, Welfare Island, N. Y.

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Address for reprint requests: Bird S. Coler Hospital, Welfare Island, New York, N. Y. 10017.

10 billion pertussis organisms, was injected into the 6 pads on the sole of the right hind foot, 0.05 ml. into each pad.

6. Eighty per cent adrenal homogenate was injected without any adjuvant, in a dose of 0.05 ml. into 5 of the 6 foot pads of the right hind foot (total amount, 200 mg. adrenal tissue). All injections were done with sterile precautions through 25-gauge disposable needles. The rats were lightly anesthetized with ether.

Some rats had the right adrenal removed 3 or 11 days before immunization. Ether anesthesia and a dorsal incision were used.

Passive Transfer

Prospective donor rats were immunized with isologous adrenal antigen, Freund's adjuvant, and pertussis vaccine as in Method 2 above. Eight or ten days later, they were anesthetized with ether and exsanguinated by needle and syringe in the aorta. The clotted blood was centrifuged and the serum was pooled. The right popliteal, inguinal, axillary, elbow, lumbar, sacral, and renal lymph nodes were enlarged and therefore presumed to drain the sites of inoculation. They were excised aseptically, pooled, cleaned of fat, rinsed in saline, and minced, and then a cell suspension was prepared by pressing the tissue through an 80-mesh stainless steel screen. The suspension was washed twice with saline by centrifugation for 10-min. periods at 200 *g* and 1–8° C. The sedimented cells were suspended in 1 ml. of saline for each donor, resieved, and injected into the dorsal penile vein of anesthetized recipients within 2–3 hr. after the donors were killed. Cell suspensions were cooled over ice at all stages.

Rats were killed for histologic study by exsanguination under ether anesthesia. Adrenals and other tissues were fixed in Bouin's solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin and with phosphotungstic acid-hematoxylin. In one experiment, the aorta was clamped at the diaphragm and adrenals were fixed by perfusion with Bouin's solution from a needle and syringe in the lower aorta.

Table 1. Allergic Adrenalitis: Roles of Antigen, Adjuvant, and Duration

Adrenal antigen	Adjuvant	Day of sacrifice	Adrenalitis incidence
Isologous	Comp. + pert.	7	0/8
		8	12/91*
		10	13/22
		14	14/14
		21	5/5*
		27	0/4
	Comp. only	10	0/2
		14	4/4*
Homologous	Comp. + pert.	8	0/4
		10	3/4
		14	4/4
	Inc. + pert.	14,20	7/9
	Inc. only	14,20	0/10
	Pert. only	20	2/3*
	None	20	0/3
Heterologous	Comp. + pert.	9,14,21	0/13
None	Comp. + pert.	14	0/5

Composite results of 7 experiments in which Lewis rats were given isologous (Lewis), homologous (other rat), or heterologous (guinea pig) adrenal homogenate, plus Freund's complete (Comp.) or incomplete (Inc.) adjuvant and/or concentrated pertussis vaccine (pert.).

* Lesions were uniformly mild.

Results

The results of the active immunization experiments are summarized in Table 1. Three of these experiments included rats which were donors of lymph node cells and serum for the passive transfers described below. Two included rats which had unilateral adrenalectomy or sham surgery before immunization; results on the remaining adrenal did not differ from and are incorporated with the results on intact animals. Guinea pig adrenal was an ineffective antigen, but other heterologous antigens have not been tried. Isologous adrenal from Lewis rats and homologous adrenal from a different rat strain or from a pool of 3 different strains were all effective. Injection of adrenal tissue with both Freund's complete adjuvant and pertussis vaccine was the most efficient way to produce adrenalitis. With this adjuvant combination, unilateral adrenalectomy or sham adrenalectomy had neither enhancing nor inhibitory activity. The effect of these operations on adrenalitis produced with weaker adjuvant combinations has not been tested. Adrenalitis was not found 7 days after inoculation but it occurred in a few rats after 8 days, in most rats after 10 days, and in all rats after 14 days. Lesions were still present after 21 days but they were very mild. No inflammation was discovered after 28 days. Not included in Table 1 are the data on 4 male L $\times$ BN F_1 hybrid rats, all of which developed adrenalitis, and 4 female Lewis rats, 3 of which developed adrenalitis after injection of Lewis rat adrenal homogenate with the two adjuvants. In additional experiments on male Lewis rats, no change was produced by doubling the dose of pertussis vaccine, or by giving twice the usual amount of both adjuvants and four times the usual amount of antigen distributed between the hind feet. A definite decrease of adrenalitis followed the injection of four times the usual dose of both adjuvants and eight times the usual dose of antigen distributed equally among all four feet.

Although pertussis vaccine was helpful, it was not essential for production of adrenalitis. Rats receiving injections of isologous antigen in Freund's complete adjuvant without the addition of a pertussis injection developed adrenalitis but of lesser severity. Two of these rats had no lesions 10 days after injection; 4 others had mild lesions 14 days after injection.

Pertussis vaccine was essential, however, when homologous adrenal antigen was emulsified in Freund's incomplete adjuvant. With antigen and incomplete adjuvant only, no lesions were obtained, while the addition of a pertussis vaccine injection produced many lesions of varying severity. The adjuvant property of pertussis vaccine was also demon-

strated when aqueous adrenal homogenate was injected without either type of Freund's adjuvant. With pertussis vaccine incorporated in the homogenate, 2 of 3 rats developed adrenalitis, albeit mild; without the vaccine, the homogenate was completely ineffective.

The fact that adrenalitis could be produced by adrenal antigen combined with either of the two adjuvants points to the adrenal tissue as the essential component of the inoculum. This was confirmed by the complete absence of adrenalitis after injections into the footpad of saline (instead of tissue) emulsified in Freund's complete adjuvant and of pertussis vaccine into the dorsum of the same foot. Nor was adrenalitis observed when isologous spinal cord, testis, or pituitary tissue was injected with the same double adjuvants.

Histopathology

The primary lesion of allergic adrenalitis was inflammatory; damage to parenchymal cells was secondary. The cortex was more severely and more often involved than the medulla. The small size and poor demarcation of the rat medulla and the occasional intermixture of cortical and medullary cells made an overlap of the inflammatory process almost inevitable. Within the cortex, none of the zones were exempt from attack, and there was no consistent site of predilection. In many mild cases, the lesions were scattered mostly through the zona fasciculata. On the other hand, zona glomerulosa, and especially the junction between glomerulosa and fasciculata, were heavily involved in severe cases. In a particular animal both adrenals were usually affected to the same degree. The inflammatory cells were aggregated into groups. Greater severity was indicated by more, larger, and denser infiltrates, by confluence, by exudation of fibrin, and by parenchymal necrosis (Fig. 1–3).

The basic lesion was a focal accumulation of mononuclear cells in the sinusoids and beneath the endothelium (Fig. 2). The infiltrate was pleomorphic and included cells with small, round, dark nuclei as in lymphocytes, large nuclei with prominent chromatin masses and nucleoli as in germinal center cells, and medium sized, vesicular, elongated, and lobated or reniform nuclei as in monocytes (Fig. 4). Cytoplasm was usually inconspicuous. In rats sacrificed 8 days, and, to a lesser extent, in those sacrificed 10 days after inoculation, there were a few polymorphonuclear leukocytes as well. After 14 days, a few rats exhibited transformation of some of the exudates into epithelioid cell granulomas; these were always in the minority and had no giant cells or vacuoles (Fig. 5). The aggregates of inflammatory cells bridged across many cords of cortical cells and seemed to engulf the intervening sinusoids (Fig. 5).

The inflammatory cells did much more than merely displace parenchymal cells. Cortical cells in the infiltrates (Fig. 2 and 4) exhibited lysis (loss of nuclear staining, dissolution of cytoplasm) or coagulation necrosis (pyknotic nuclei, hypereosinophilic cytoplasm). On the periphery of infiltrates and especially of epithelioid cell granulomas, the cortical cells were excessively and irregularly vacuolated (Fig. 1 and 2). Small foci of similar appearance were also present without adjacent inflammation. The most severe lesion was focal necrosis extending through several adjacent cell cords, usually of the fasciculata, with a few polymorphonuclear leukocytes, abundant fibrin, and complete destruction of pre-existing structures (Fig. 1 and 3). These areas were usually in or just below zones of confluent inflammation.

Adrenalitis produced with the aid of pertussis vaccine, or pertussis vaccine and Freund's incomplete adjuvant, or Freund's complete adjuvant without pertussis vaccine, had inflammatory exudates identical with those described above following the use of both complete adjuvant and pertussis vaccine. Focal parenchymal necrosis was seen only with the latter adjuvant combination. Hemorrhage was not conspicuous, even in rats with adrenalitis that were given large doses of heparin (40 units per gram intravenously) 7 hr. before sacrifice.

The lesions observed in rats corresponded to the mononuclear infiltrates described in guinea pig and rabbit adrenalitis[3-7] except that plasma cells were not conspicuous and there was no consistent zone of predilection. Infiltrates of predominantly polymorphonuclear character, seen in 3 rabbits and 1 guinea pig by Barnett, Dumonde, and Glynn,[7] were not observed. Parenchymal necrosis, observed in guinea pigs by Waksman,[12] was found in a few rats.

Other Organs

Popliteal lymph nodes of rats immunized by Method 2 had oil vacuoles, acid fast bacilli, suppuration, granulomas, and thrombosis of sinusoids caused by absorption of adjuvants.[8] Many of these animals developed polyarthritis, ear nodules, and hemorrhagic necrosis of the spleen after 11 or more days. These lesions were due to "adjuvant disease"[13] and had no relation to adrenalitis. Adjuvant disease occurred after injection of adjuvants without adrenal tissue or with material heated at 60° C. for 1 hr, whereas adrenalitis was absent or minimal, respectively, under these conditions. Conversely, adrenalitis but not adjuvant disease occurred in rats immunized by Method 4 (no tubercle bacilli) or by passive transfer of adrenal-immunized lymph node cells.

No lesions were found in the pituitary, pancreas, liver, or kidneys. A

few insignificant lesions were found in occasional thyroids, testes, and epididymes and in one spinal cord. Chronic murine pneumonia occurred in 10–25% of both experimental and control animals and had no relation to adrenalitis.

Passive Transfer

In the first experiment, 16 donors were immunized with Lewis adrenal tissue, Freund's complete adjuvant, and pertussis vaccine. Their draining lymph nodes and serum were removed 10 days later, when more than half had severe adrenalitis. The cell suspension was injected into 4 recipients, 2 of which had been subjected to unilateral adrenalectomy 10 days before ($.6 \times 10^9$ cells per dose, donor to recipient ratio = 4:1). Recipients were sacrificed 7 days after passive transfer. Only one nonoperated recipient had a minor lesion in one adrenal. Two additional unilaterally adrenalectomized rats were given 18 ml. of donor serum each, but they did not have any lesions.

In the second and third experiments, 40 and 48 donors were prepared in similar manner, but were used 8 days later, when only 3 and 8, respectively, had adrenalitis. Unilateral adrenalectomy of recipients was not performed because of the lack of benefit demonstrated above. The donor lymph node cells were injected into two recipients at a dose of $.35 \times 10^9$ cells (donor to recipient ratio = 4:1) and, again, only one recipient had a minor lesion in one adrenal. However, injection of 1.4×10^9 or 2.1×10^9 cells (donor to recipient ratio = 16:1) gave bilateral adrenalitis in all 5 recipients. This was severe in 2 recipients and moderate in 1, sacrificed after 7 days, and mild but definite in 2 recipients sacrificed after 6 days. The lesions were identical with those described after active sensitization. Other organs (pituitary, thyroid, testes, pancreas, liver, spleen, kidney, and central nervous system) had no significant lesions. Thirty milliliters of donor serum failed to cause lesions in either of 2 additional recipients.

It is clear that the success of passive transfers was due to the large doses of cells, and possibly also to early sacrifice of donors. There was a remote possibility that the large numbers of donor cells carried with them sufficient adrenal antigen and adjuvants to cause adrenalitis in recipients by active immunization. However, the occurrence of adrenalitis 6 and 7 days after transfer made this unlikely, inasmuch as the incubation period for active immunization was not less than 8 days. Even at 8 days, the few actively immunized rats that developed adrenalitis had only mild lesions (Table 1). Furthermore, preliminary experiments indicate that passive transfer can be accomplished in 24 hr. with much smaller doses of cells, provided that nonspecific damage has been inflicted previously on the

target adrenal. Because of the apparent thermal lability of the adrenal antigen, the injection of heat-killed cells would not exclude the possibility of antigen carry-over. Transfers with killed or inactivated cells have not yet been attempted.

To prove that passive transfer of adrenalitis was a specific response to adrenal-immunized lymphoid cells, the last experiment was repeated in an identical manner except that the anterior lobes of Lewis rat pituitaries were substituted for the adrenal antigen. The donor lymph-node-cell suspension (3×10^9 cells per dose) caused no adrenal lesions in any of the 3 recipients (donor to recipient ratio = 16:1).

Discussion

The results reported here in rats compare favorably with those obtained in other species[3-7] in that repeated inoculations and unilateral adrenalectomy were not required, and lesions appeared as early as 8 days after inoculation and were high in incidence by 10 or 14 days. The adjuvant activity of pertussis vaccine and Freund's adjuvant, either alone or in combination, has precedent in work on EAE.[8] However, EAE can be produced with the aid of Freund's incomplete adjuvant alone[9] or even with no adjuvant at all.[10] These differences are not due to intrinsic adjuvant activity in neural tissue,[11] but may be due to greater antigenicity of neural tissue or the presence of inhibitors (perhaps corticosteroids) in adrenal tissue.

Adrenalitis has been produced in guinea pigs and rabbits with autologous, homologous, and heterologous adrenal antigens.[3-7] Therefore, the ineffectiveness of the single heterologous antigen tried in rats is not conclusive evidence for the species-specificity of the rat adrenal antigen.* Inasmuch as Lewis rats are isohistogenic, the production of adrenalitis with isologous Lewis rat antigen can be considered the equivalent of autosensitization in guinea pigs[5] and rabbits.[4]

The success of passive transfer can be attributed to the employment of inbred isohistogenic animals as donors and recipients. This ensured survival of donor cells in the new host.[14,15] Nevertheless, the number of cells required for passive transfer was higher and the incubation period longer than we have observed in passive transfer of EAE.[15] These differences are probably due to the more intense immunization of donors obtained with neural antigen. Passive transfer with living lymphoid cells has also been accomplished with thyroiditis,[16,17] neuritis,[18] aspermatogenesis,[19] and adjuvant disease[13] as well as EAE.[14,15,18]

Adrenalitis in rats appears to conform to the definition of an auto-

* Recently, we have produced adrenalitis in rats with dog and rabbit adrenal tissue.

immune disorder.[12,14] Its allergic character is indicated by production with specific antigen, by the latent period, the abatement of the reaction with the passage of time, the specificity of the response, and passive transfer with cells. Antiadrenal antibodies are produced concomitantly.[4,6,7] Yet to be demonstrated are specific desensitization or immunologic tolerance.

The mononuclear character of the exudate and the successful transfer with lymphoid cells suggest that adrenalitis is related to delayed or cellular hypersensitivity.[12] However, failure to achieve passive transfer with serum does not exclude a role for a humoral antibody, because the serum may have lost antibody in the donor's adrenals[20] or may have been collected at an inappropriate time or administered in inadequate dosage.

Summary

Allergic adrenalitis was produced in Lewis rats by a single injection of isologous or homologous adrenal tissue aided by adjuvants. The most efficient procedure required both Freund's complete adjuvant and pertussis vaccine, but adrenalitis was produced with the aid of Freund's complete adjuvant only, pertussis vaccine only, or Freund's incomplete adjuvant plus pertussis vaccine. The incubation period was 8–10 days. Adrenalitis was maximum at 14 days and waning at 21 days. Both cortex and medulla had many aggregates of mononuclear inflammatory cells, some of which evolved into epithelioid cells. In the cortex, the inflammation caused lysis and coagulation necrosis of small groups of cortical cells. In the most severe instances, there was diffuse confluent inflammation with more extensive but still focal necrosis.

Adrenalitis was transferred passively from actively immunized donors to normal recipients through the agency of living lymph node cells. These data provide additional evidence of the autoimmune character of adrenalitis, and, furthermore, contribute to its classification with other forms of delayed or cellular hypersensitivity.

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[*Illustrations follow*]

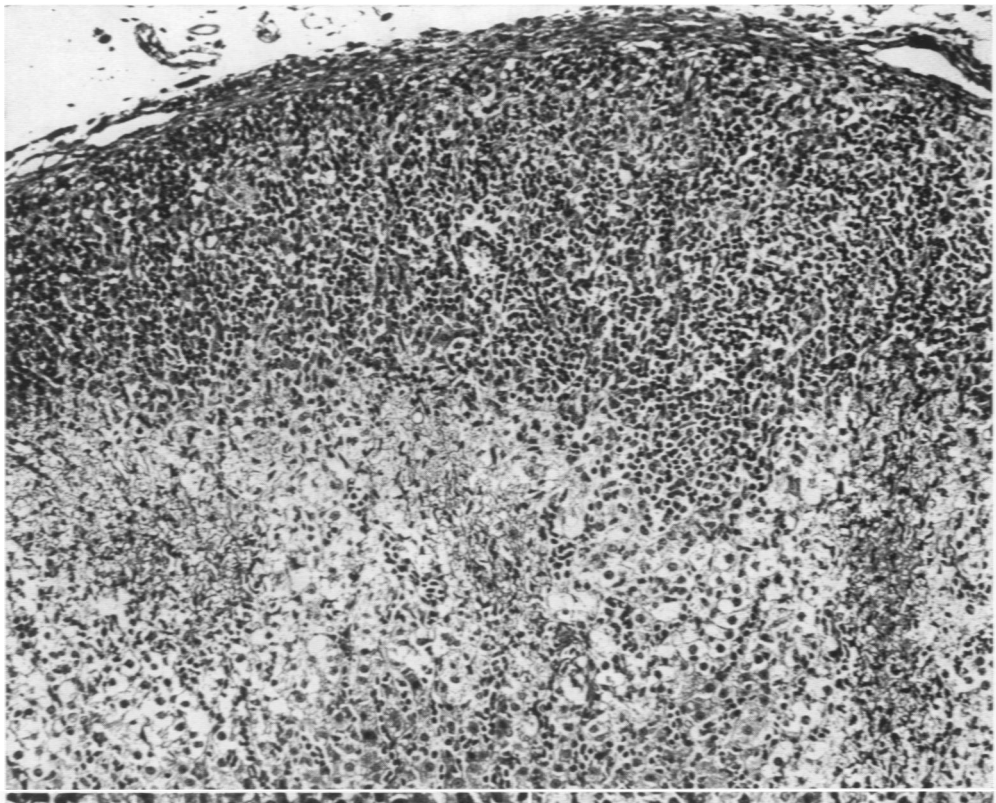
Legends for Figures

All photomicrographs depict adrenal cortex from rats with allergic adrenalitis.

Fig. 1. (Upper half). Dense, confluent inflammation of zona glomerulosa and upper fasciculata obliterates all pre-existing structures. (Lower half). There is excessive vacuolation and pallor of fasciculata cells with 3 darker-staining foci of necrosis. Hematoxylin and eosin. × 130.

Fig. 2. Linear columns of inflammatory cells fill sinusoids. Aggregates of inflammatory cells (right of midline) have destroyed cortical parenchymal cells. Still visible are groups of cortical cells which exhibit various stages of lysis (arrows); some have inflammatory cells in their cytoplasm. Cortical cells near top are excessively vacuolated. Hematoxylin and eosin. × 275.

Fig. 3. Abundant fibrin and inflammatory cells in a focus of necrosis. Phosphotungstic acid-hematoxylin. × 170.



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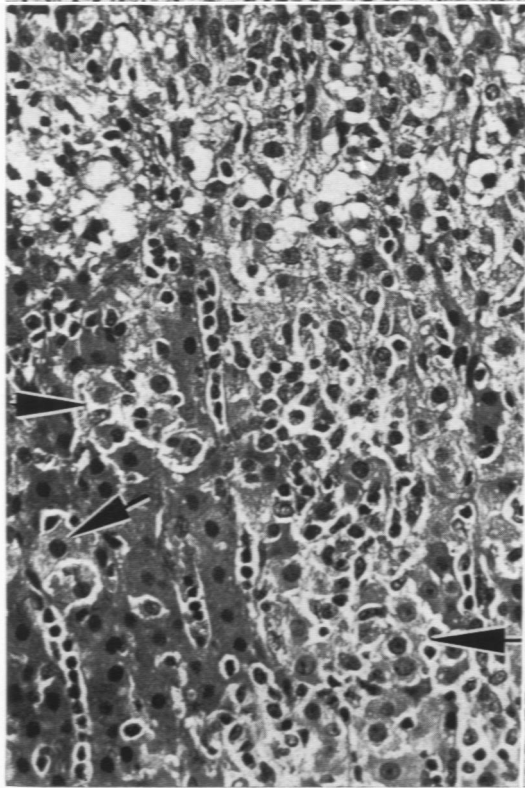

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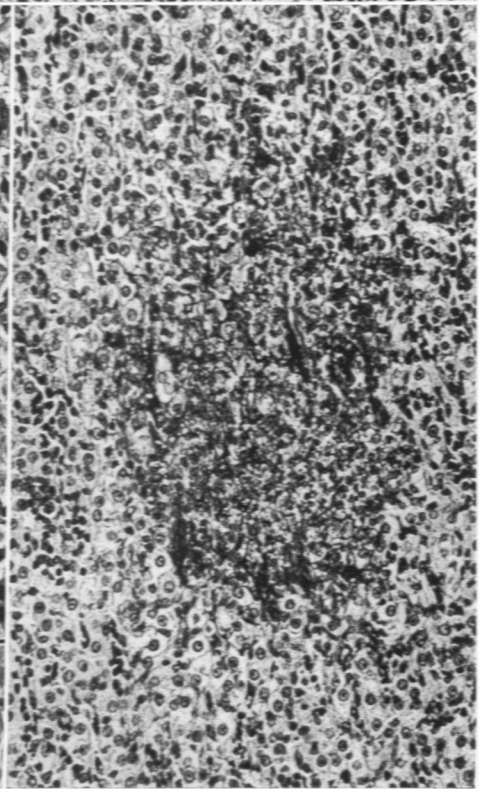

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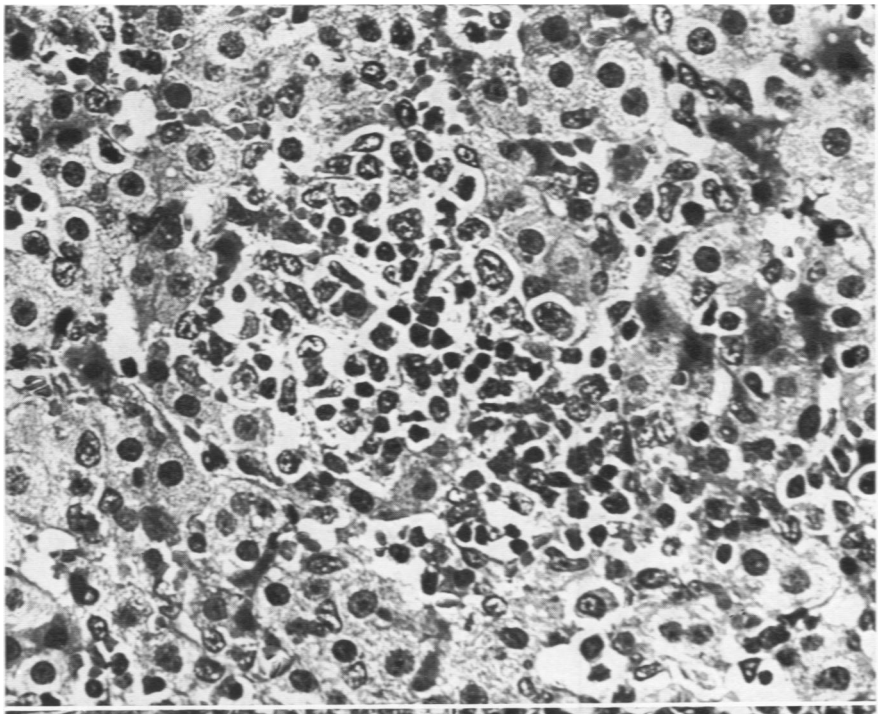
Fig. 4. Mononuclear infiltrate has pleomorphic character. Cortical cells on periphery of aggregate exhibit coagulation necrosis and cell lysis. Hematoxylin and eosin. × 440.

Fig. 5. Infiltrate containing lymphocytes and epithelioid cells (between arrows). Granuloma has engulfed and obliterated several cell cords and intervening sinusoids. Surrounding sinusoids are distended and almost empty because perfusion fixation was employed. Hematoxylin and eosin. × 290.



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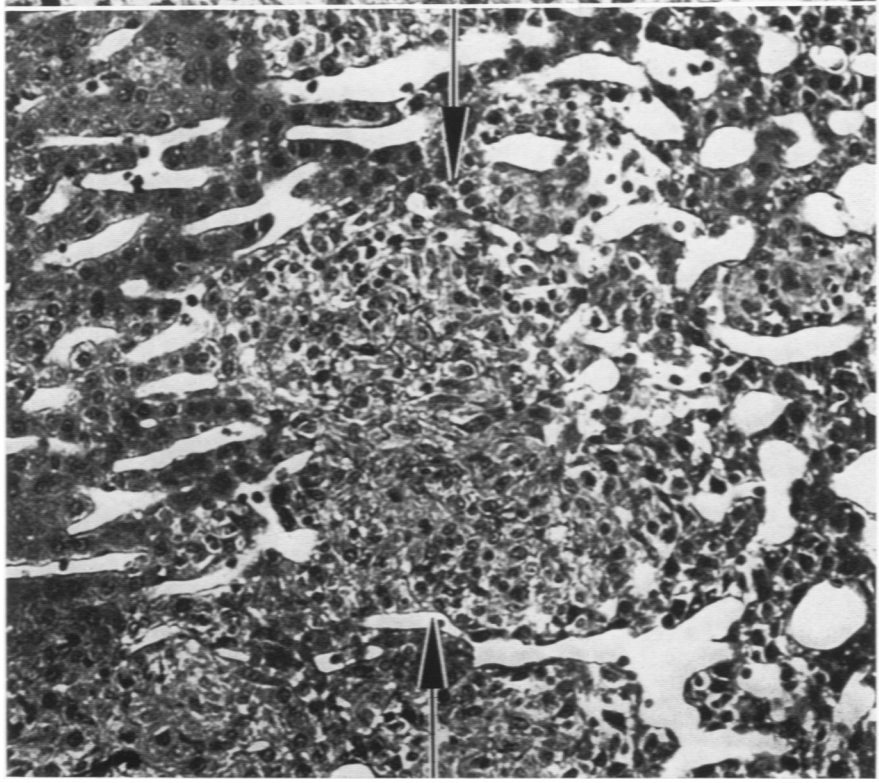

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