Immune Responses of the Protein-Deficient Guinea Pig to BCG Vaccination

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Two groups of guinea pigs were maintained on high-protein and low-protein diets and immunized with intradermal BCG. Protein deficiency was accompanied by marked inhibition of local and systemic immune responses: a) The BCG nodule was poorly formed. There was marked delay and deficiency in the mobilization of macrophages. b) The draining lymph node was atrophic and showed little or no proliferation of lymphoid cells in the paracortical area. Macrophage accumulation occurred late but became diffuse and marked, in contrast to its consistent scarcity in the BCG nodule. c) In either location epithelioid cell transformation was retarded. Well-formed mature epithelioid cell granulomas were not seen. d) Bacilli persisted for a long time in the skin and lymph node lesions, e) Tuberculin sensitivity was greatly impaired in one-fifth of animals and absent in others. These findings were suggestive of macrophage dysfunction and depression of cell-mediated immunity to BCG in the protein-deficient guinea pig (Am J Pathol 72: 489–502, 1973).

PROTEIN MALNUTRITION and low economy are associated with a high incidence of tuberculosis. This is evident in clinical, epidemiologic and autopsy studies.[1–11] Tuberculosis induced in protein-deficient animals has, however, yielded conflicting results. The adverse effect of dietary protein deficiency has been demonstrated in some experiments,[12–17] but no demonstrable effect has been seen in others.[18,19,20]

In the past, diminished antibody production has been suggested as a factor in the increased susceptibility to tuberculosis in protein depletion.[3,16] The role of antibody in this infection is, however, uncertain,[21,22] On the other hand, cell-mediated immunity appears to play a greater role.[23] In malnourished children, tuberculin sensitivity, a cell mediated immune response, remains greatly impaired following natural infection or BCG vaccination.[7,24–26] Passive transfer studies show a defect in induction rather than expression of tuberculin allergy in these children.[27] The prevalent pattern of infection in protein-calorie malnutrition further suggests a marked depression of cell-mediated immunity.[28]

The early inductive phase of cell-mediated immunity, which ap-

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pears essential for effective development of antituberculous immunity and tuberculin allergy, has not been studied in detail in conditions of protein-deficient nutrition. Animal experimentation provides an excellent opportunity for this purpose and has been utilized in the present investigation.

Materials and Methods

Eighty-eight guinea pigs of either sex, weighing between 280 and 300 g, were divided equally into high-protein (HP) and low-protein (LP) groups. They were housed in batches of 4 per cage and fed isocaloric HP and LP diets, respectively, depending on the latter consumption. This was supplemented with 20 g of carrot per animal per day. The HP diet consisted of 20% casein and the LP diet, 2% casein. The diets were otherwise well balanced and contained adequate amounts of minerals and vitamins, including vitamin A and vitamin C (Table 1).

After 5 weeks of experimental diet, each animal was given, intradermally, a total of 0.2 mg of BCG (BCG Laboratory, Madras, India) in 0.2 ml of 0.01 M phosphate buffer, pH 7.2. Injections were made in the upper and lower halves on the dorsum of left ear. The right ear was similarly treated with buffer alone.

Development of the BCG nodule and reactions in the draining preauricular lymph node were studied in 5 to 6 animals of either group on days 0, 1, 3, 5, 7, 10, 14, 21, 28 and 35. The radius(r) of the BCG nodule was measured by centimeter scale and thickness (t) by dial calipers (Schnelltaster, Germany), and volume determined from the formula $4/3\ \pi r^2 t$ for an oblate ellipsoid.[29] The lymph node was weighed in the Mettler balance. The tissues were fixed in neutral buffered formalin and sectioned after routine processing. The cellular reaction was studied in hematoxylin and eosin-stained sections, and bacilli were demonstrated with modified Ziehl-Neelsen (carbolfuchsin-hematoxylin) stains. The lymph node sec-

Table 1—Percentage Composition of Guinea Pig Diets*

Constituents	High Protein (%)	Low Protein (%)
Casein	20	2
Sucrose	30	48
Powdered sago	35	35
Ground nut oil	8	8
Vitamin mixture†	2	2
Salt mixture‡	4	4
Choline chloride	0.8	0.8
Vitamin C	0.2	0.2

* To 100 g of each diet, 1200 units of vitamin A, 200 units of vitamin D and 6 mg of vitamin E were added.

† Vitamin mixture: thiamin, 300 mg; riboflavin, 500 mg; pyridoxin, 300 mg; folic acid, 100 mg; vitamin K, 100 mg; vitamin B_{12}, 10 mg; biotin, 10 mg; calcium pantothenate, 1 g; nicotinic acid, 1 g; PABA, 1 g; inositol, 5 g; sucrose, 990.68 g.

‡ Salt Mixture: $CaCO_3$, 300 g; K_2HPO_4, 320 g; $CaH\ PO_4 \cdot 2H_2O$, 80 g; $MgSO_4 \cdot 7H_2O$, 100 g; NaCl, 160 g; $Fe(C_6H_5O_7) \cdot 6H_2O$, 36 g; $MnSO_4 \cdot 4H_2O$, 3.4 g; $ZnCl_2$, 200 mg; $CuSO_4 \cdot 5H_2O$, 350 mg; KI, 50 mg.

tions were treated in addition, with methyl green-pyronin. Findings were arbitrarily graded as +, ++ and +++, indicating mild, moderate and marked reactions, respectively.

The tuberculin test was performed in 10 to 12 animals of either group at weekly intervals. Both flanks were gently shaved and cleaned dry. On the left flank 100 tuberculin units (TU) pruified protein derivative (PPD) (BCG Laboratories, Madras) were injected intradermally in 0.1 ml saline. The right flank was injected with 0.1 ml saline alone. At 24 and 48 hours the diameter and thickness of the indurated erythematous lesion were measured and volume calculated like the BCG nodule. Hematoxylin and eosin-stained sections of the lesional tissue were examined for dermal reactions to tuberculin.

At the end of experiment, animals were etherized and exsanguinated. Blood was collected for estimation of serum proteins. Pieces of liver, lung, pancreas, thymus, spleen, and cervical and mesenteric lymph nodes were obtained for microscopic examination.

Results

General Effects of Protein Deficiency

In both groups, the daily dietary consumption of each animal was, approximately, 12 g initially and 8 g toward the end; this was in addition to the 20-g carrot supplement.

Animals receiving the HP diet grew slowly and weighed 340 to 365 g in 10 weeks. They remained healthy and active. Serum protein levels were 7.08 g/100 ml (SE = 0.12), with albumin levels of 4.02 g/100 ml (SE = 0.11). The lymphoid tissues were cellular, and the organs unremarkable. Animals on the LP diet did not grow at all; rather, there was a progressive fall in body weight which was rapid at first and slowed down later. Towards the end the animals weighed 170 to 180 g. Some of them looked relatively inactive and unwell; about one-tenth died. The serum protein level was 5.71 g/100 ml (SE = 0.18), with albumin levels of 2.90 g/100 ml (SE = 0.13). Marked involution of the lymphoid tissue, fatty liver and atrophic pancreas also characterized these animals.

Development of the BCG Nodule

HP Guinea Pigs

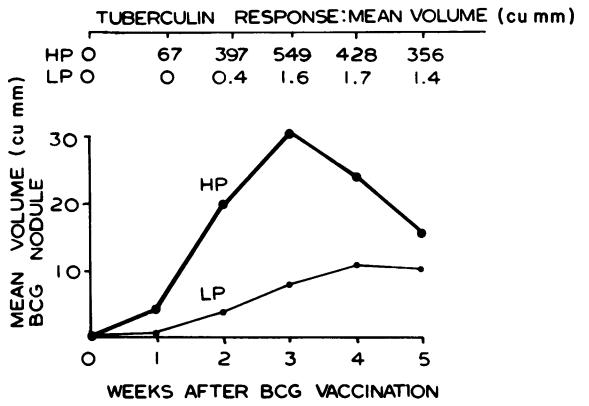
The inoculated site appeared erythematous on day 1 and nodular on day 7. The nodule increased rapidly and measured 30 cu mm in 3 weeks (Table 2; Text-figure 1). It ulcerated frequently and healed rapidly, leaving behind a flat and firm lesion at the end of 5 weeks.

Microscopically, an acute inflammatory exudate consisting of numerous neutrophils and few macrophages was seen on day 1. Acid-fast bacilli (AFB) were numerous (Text-figure 2). A diffuse accumulation of macrophages containing acid-fast bacilli was seen on

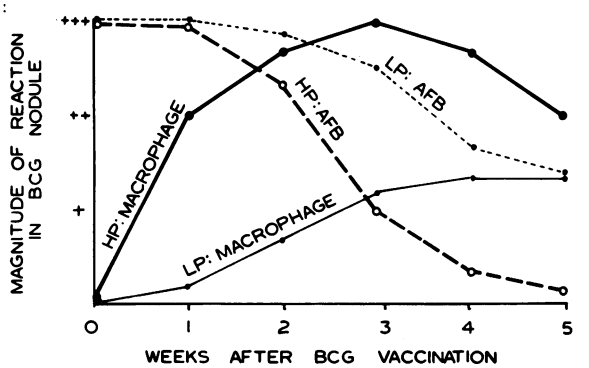
Table 2— Quantitative Estimation of BCG Nodule, Draining Lymph Node and 24-Hour Tuberculin Response

Time after BCG	Exp group	BCG nodule volume (cu mm; mean ± SE)	Lymph node weight (mg; mean ± SE)	Tuberculin response volume (cu mm; mean ± SE)
0-Week	HP	0	12.25 ± 0.47	0
	LP	0	4.10 ± 0.27	0
1-Week	HP	4.00 ± 0.35	20.20 ± 1.02	67.4 ± 4.82
	LP	0	6.43 ± 0.59	0
2-Week	HP	20.15 ± 0.92	32.70 ± 1.71	397.3 ± 18.10
	LP	3.13 ± 0.10	8.43 ± 0.59	0.4 ± 0.04
3-Week	HP	30.29 ± 1.54	49.88 ± 2.14	548.6 ± 24.70
	LP	8.00 ± 0.50	14.12 ± 0.60	1.6 ± 0.05
4-Week	HP	24.27 ± 1.06	46.00 ± 1.73	428.3 ± 17.80
	LP	11.12 ± 0.27	16.08 ± 0.47	1.7 ± 0.07
5-Week	HP	15.30 ± 0.91	36.50 ± 1.32	355.6 ± 16.20
	LP	10.15 ± 0.40	15.15 ± 0.40	1.4 ± 0.07

day 7. Further accumulation led to the formation of a compact nodule of macrophages. Immature epitheliod cells (macrophages with intercellular cytoplasmic connections) containing diminished num-



TEXT-FIG 1—Growth and regression of the BCG nodule and tuberculin response of the HP and LP guinea pigs at weekly intervals. See Table 2 for details.



TEXT-FIG 2—Mobilization of macrophages into and disappearance of AFB from the BCG nodule. (Mild accumulation of macrophages or rarely detectable AFB, +; moderate accumulation of macrophages or easily demonstrable AFB, ++; marked macrophage accumulation or numerous AFB, +++).

ber of bacilli made their appearance on day 14 (Figure 1). Whorls of mature epithelioid cells forming tubercles and frequently containing Langhans giant cells were seen during 3 to 5 weeks (Figure 2). There was moderate lymphocytic infiltration and fibroplasia. Acid-fast bacilli were rare and often undetectable.

The overlying epidermis showed marked acanthosis at 1 to 2 weeks (Figure 1). The midportion frequently underwent necrosis and ulceration. The remaining portion continued to show moderate acanthosis during 3 to 5 weeks.

LP Guinea Pigs

A transient erythema was seen on day 1, but no appreciable lesion developed until day 7. On day 14 a small nodule was observed which slowly increased in size and measured 11 cu mm at 4 weeks. There was slight reduction at the end of 5 weeks. Ulceration of the nodule was rare.

Histologically, a moderate number of neutrophils and rare macrophages were seen on day 1 amidst numerous AFB. The neutrophils continued to accumulate until day 3 and persisted in significant numbers until day 7. Macrophage accumulation was very slow and only a scattered number was seen on day 14 (Figure 3). The further increase was not appreciable. Most macrophages were discrete, with bulky eosinophilic or reticulated cytoplasm. There were few foreign body giant cells, and Langhans giant cells were rare. During 4 and 5 weeks after BCG injection, foci of immature epithelioid cells containing AFB were encountered, mature epithelioid cells were rare, and tubercle formation was not seen (Figure 4). In one-half of the animals, a central area of necrosis containing numerous AFB was present. Lymphocytic infiltration in the lesion was sparse, and fibroplasia poor.

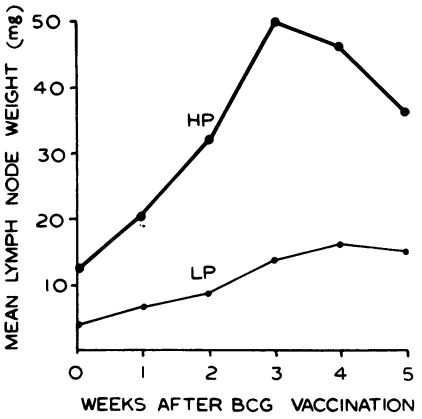
The overlying epidermis showed mild acanthosis. Epidermal necrosis and ulceration were rare.

Reactions in the Draining Lymph Node

HP Animals

The preauricular lymph node weighed 12 mg on day 0. It became progressively enlarged and attained a peak weight of 50 mg in 3 weeks (Text-figure 3). The cut surfaces revealed pale yellow homogenous tissue and occasional foci of necrosis. There was moderate reduction in size during 4 and 5 weeks.

Sections showed well-formed cortex with moderately active ger-



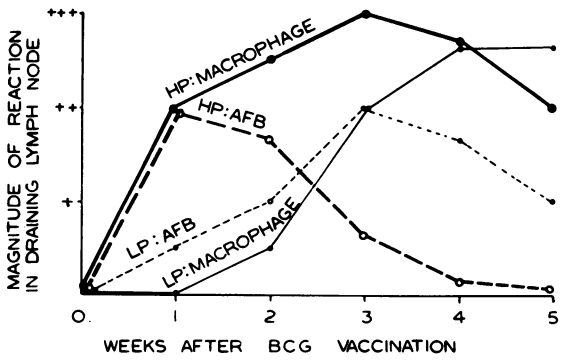
TEXT-FIG 3—Enlargement of the draining lymph node in HP and LP animals after intradermal BCG. See Table 2 for details.

minal centres and a cellular paracortex containing lymphoid cells. Clusters of macrophages with ingested bacilli appeared on day 3 and formed a prominent feature on day 7 (Figure 5; Text-figure 4). The germinal centres appeared active, and the paracortex was enlarged, with pyroninophilic cells showing frequent mitoses. Continuously migrating macrophages occupied a large portion of the lymph node on day 14. Well-formed epithelioid cell granulomas were seen in 3 to 4 weeks. Caseation necrosis was present in about half of the animals (Figure 6).

LP Animals

The draining lymph node was markedly atrophic and weighed 4 mg on day 0. It showed moderate increase and weighed 16 mg in 4 weeks. No further increase was observed.

Histologic examination revealed pronounced depletion of lymphoid cells. The cortex was thin, with few lymphoid follicles; active germinal centres were not seen. The paracortical area was sparsely



TEXT-FIG 4—The phenomena of macrophage mobilization and bacillary appearance and disappearance from the draining lymph node. For significance of +, ++, and +++ refer to Text-figure 2.

populated with lymphoid cells. Macrophages were not seen in significant numbers until day 7. AFB, however, were seen singly or in clusters lying free in sinuses and were rarely seen in macrophages. Germinal centres were occasionally seen, and the paracortical area appeared cellular with mildly pyroninophilic cells showing rare or no mitoses (Figure 7). Macrophages began to appear on day 10; a significant number of them were observed occupying cortex and paracortex on day 14. A diffuse, scattered accumulation of macrophages, forming foci of immature epithelioid cells and containing AFB, was observed during 3 and 4 weeks (Figure 8). Mature epithelioid cell formation was, however, rare. Granuloma formation and caseation necrosis were not seen.

Tuberculin Responses

HP Group

At the end of 1 week, a small indurated erythematous lesion was seen at 24 hours after the intradermal injection of 100 TU tuberculin, and it appeared diminished at 48 hours. Maximal response was seen in 3 weeks; at this time, the 24-hour lesion measured 18 mm in diameter and 549 cu mm in volume. The intensity of reaction appeared slightly diminished at 48 hours. Microscopically, a diffuse perivascular infiltrate of mononuclear cells was seen in the dermis.

LP Group

No response to the intradermal injection of 100 TU tuberculin was seen during the first week. A small area of erythema and induration was seen in about one-fifth of animals during 2 to 5 weeks. It measured 3 to 4 mm in diameter and 1 to 3 cu mm in volume at 24 hours and disappeared at 48 hours. Microscopically, rare histiocytes were observed in the dermis. In the majority of animals the tuberculin sensitivity was absent.

Discussion

Guinea pigs have been used in this study because of their demonstrable susceptibility to tuberculosis and ability to develop tuberculin sensitivity.[30] The adverse effects of vitamin A and vitamin C deficiency, observed in clinical and experimental tuberculosis,[3,31–33] have been obviated in this study by incorporating them into the diets in adequate amounts (Table 1).

Use of different animals in experimental tuberculosis might account for conflicting results obtained in the past. The rat is particularly unsuitable, as it is relatively resistant to both tuberculous

infection[16] and protein-deficient nutrition.[34] Only in the study of Koerner *et al*,[15] has this animal, fed low protein diet, shown poor localization of tuberculous lesions, wide dissemination and persistence of large number of bacilli in the epithelioid cells. This has not been found in other studies.[18–20] Mice, hamsters and birds appear to be relatively more susceptible to tuberculosis under protein starved conditions.[12–17] The guinea pig is very vulnerable to protein depletion.[34] Ratcliffe and Merrick,[17] who used this animal at different levels of protein nutrition, found no difference in the evolution of pulmonary tuberculosis within 75 days of droplet infection with virulent bacilli. Beyond this time the lesions healed in high protein animals, but continued to remain active in low protein animals and showed ill-defined, irregularly expanding tubercles which contained numerous bacilli and large areas of necrosis and formed secondary tubercles in the lung. With less virulent bacilli, no difference was observed until 180 days, but extrapulmonary lesions healed more rapidly in the high protein than in the low protein animals. Sriramachari and Gopalian[20] also used guinea pigs and induced tuberculosis by intravenous inoculation of HRV 37 but did not find any difference between the animals at different level of protein nutrition.

Infection with virulent bacilli, in these studies, might have obscured subtle differences in the host response at different levels of protein nutrition. This could be overcome by intradermal inoculation with attenuated mycobacteria BCG, which is used extensively for active immunization against tuberculosis in human beings. Evolution of the primary complex in the early inductive phase of the immune response could be observed in detail by this procedure. Bryceson and Turk[29] have recently studied this aspect in normal and antilymphocyte-serum (ALS)-treated guinea pig. The present study deals with the high-protein and low-protein guinea pigs.

In the HP guinea pig, the BCG nodule was well formed and showed diffuse accumulation of macrophages and numerous ingested bacilli at 1 and 2 weeks. The macrophages were soon transformed into immature and mature epithelioid cells. Well-formed granulomas with rarely detectable AFB were seen at 3 and 4 weeks. There was marked epidermal acanthosis and frequent ulcertion followed by rapid healing. The draining lymph node was cellular and showed large clusters of macrophages with ingested bacilli at 1 and 2 weeks. There was lymphoid cell proliferation in the paracortical area. Well-formed epithelioid cell granulomas with frequent caseation necrosis were observed at 3 and 4 weeks. Tuberculin

sensitivity appeared early and remained persistent; microscopically, it was characterized by diffuse dermal accumulation of mononuclear cells.

Protein malnutrition profoundly altered the responses of the guinea pig to BCG. The BCG nodule was poorly formed and showed only scattered accumulation of macrophages at 1 and 2 weeks. Bacilli, ingested and uningested, were numerous. The macrophages continued to be scarce and remained largely discrete, with focal formation of immature and mature epithelioid cells at 4 and 5 weeks. Intracellular bacilli were seen in fair numbers. There was mild epidermal acanthosis and rare ulceration, followed by slow healing. The draining lymphnode was atrophic and showed mild or no proliferation of lymphoid cells in the paracortex at 1 and 2 weeks. Macrophages were inconspicuous and bacilli detected free in the sinuses. After 2 weeks, however, there was large and progressive accumulation of macrophages which diffusely occupied the lymph node. The macrophages remained discrete or formed focal immature and mature epithelioid cells. Intracellular AFB were easily detectable. Well-formed granulomas and caseation necrosis were not seen. Tuberculin sensitivity, which appeared late, remained greatly impaired in about one-fifth of animals and absent in others. Microscopically, few mononuclear cells were observed in the dermis.

Macrophages play a crucial role in mycobacterial infection.[5,23,35,36] Their early influx and continued accumulation is augmented by local proliferation.[35,37,38] They ingest the bacilli, become stimulated and undergo adaptive changes with increase of hydrolytic enzymes.[38,39] Adaptive changes are accelerated by development and operation of cell-mediated immunity.[23] This results in rapid digestion and disposal of intracellular bacilli and formation of epithelioid cells and tubercles.

The delayed and defective mobilization of the macrophages in protein deficiency might result from a low circulating pool. Impairment of cellular proliferation[40] might further account for consistent scarcity of these cells in the BCG nodules. Their delayed and diffuse accumulation in the draining lymph node, however, is in marked contrast and might have resulted from local recruitment and persistent drainage from the skin lesion. The macrophages in the skin and lymph node were apparently defective, as observed earlier by Yang and Skinsness[41] in protein-deficient mice, and incapable of optimal stimulation and adaptation to deal with the ingested bacilli. Impairment of cell-mediated immune responses might have further hampered macrophage function. The result was long continued persistence of intracellu-

lar bacilli, focal formation of epithelioid cells and absence of well-formed granulomas.

The interrelationship between the accumulation of macrophages and disappearance of bacilli is clearly seen in Text-figures 2 and 4. In the HP guinea pig, this is apparent after 1 week of vaccination, and it forms a pronounced feature. Disappearance of bacilli is associated with transformation of macrophages into mature epithelioid cells and tubercles. In the LP guinea pig, on the other hand, macrophage accumulation is slow, and bacilli start disappearing in significant numbers only after 3 weeks. This is associated with formation of immature and rarely mature epitheliod cells, but no well-formed tubercles are seen. Rapid macrophage mobilization and granuloma formation are manifest attempts to localize the mycobacterial infection. Retardation of this process in the protein-deficient guinea pig has apparently allowed the BCG to spread beyond the draining lymph node. Absence of detectable lesions in the distant organs might, however, reflect limited multiplication of BCG in this animal as in man.[29] With virulent bacilli, ill-defined tubercles and disseminated tuberculosis have been observed, as discussed earlier, in the protein-deficient rat and guinea pig.[17]

Lurie *et al*[42] observed that a well-formed BCG nodule was a fair index of host resistance and found it poorly formed in the susceptible rabbit. Bryceson and Turk[29] made a similar observation in the ALS-treated guinea pig. In this animal, moreover, well-formed epithelioid cell tubercles were not observed; there was lack of lymphoid cell proliferation in the paracortical area of the lymph node and pronounced impairment of tuberculin response. Findings in the protein-deficient guinea pig closely resembled these observations and were suggestive of dysfunction of the macrophage and depression of cell-mediated immunity.

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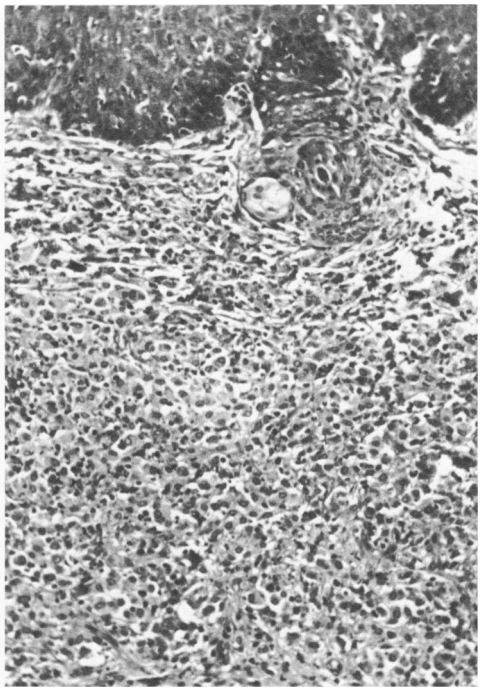

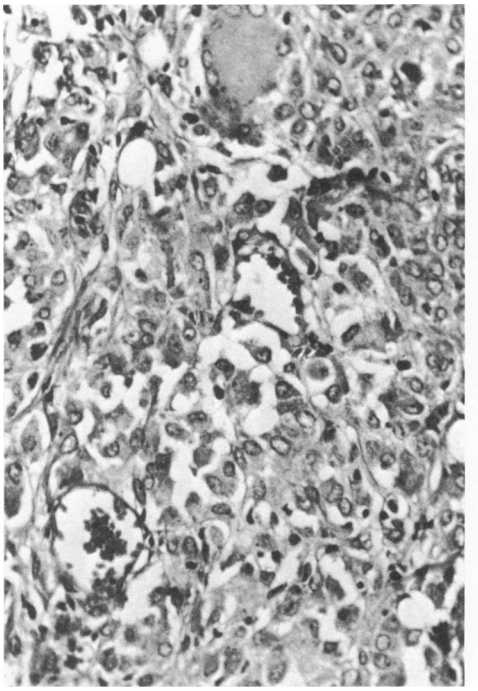

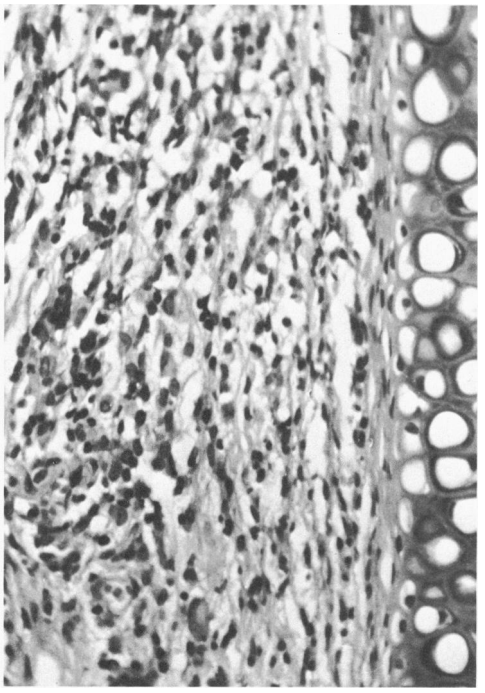

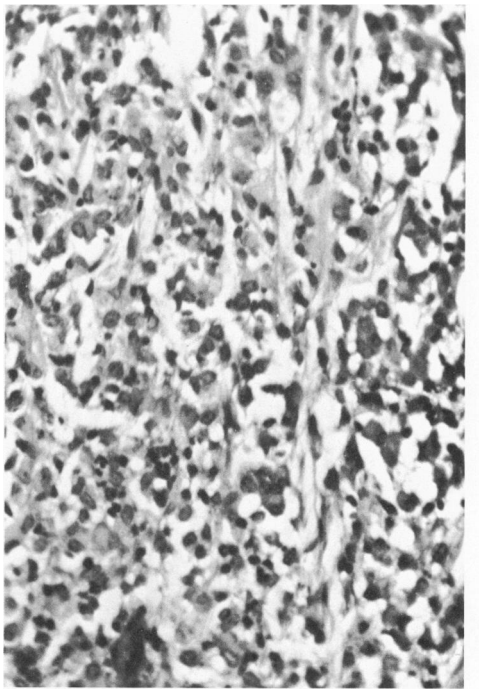

Fig 1—Fourteen-day-old BCG lesion in a HP guinea pig, showing diffuse accumulation of macrophages in the dermis and prominent acanthosis (H & E, × 120). **Fig 2**—Dermal lesion in a HP animal 28 days after BCG. Well-knit whorls of large mature epithelioid cells forming tubercles and Langhans giant cells are seen (H & E × 240). **Fig 3**—Fourteen-day-old BCG lesion in the LP guinea pig, showing scattered accumulation of macrophages in the dermis close to the auricular cartilage (H & E, × 240). **Fig 4**—Dermal lesion in an LP animal 28 days after BCG, showing discrete macrophages with bulky eosinophilic or reticulated cytoplasm. Foci of immature epithelioid cells are also seen. Well-formed tubercles are absent (H & E, × 240).

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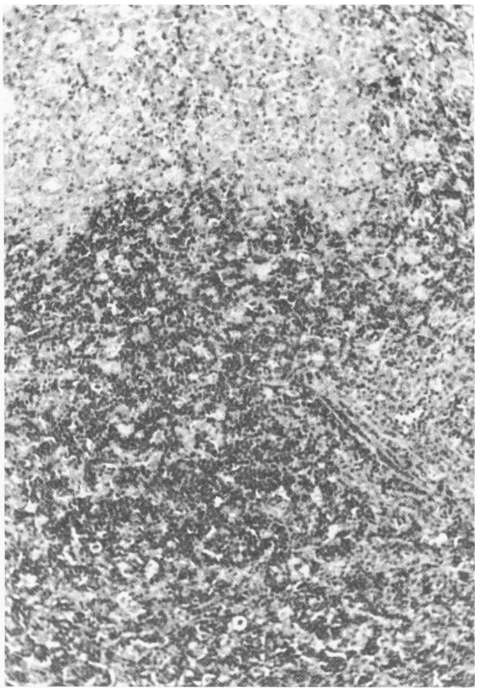

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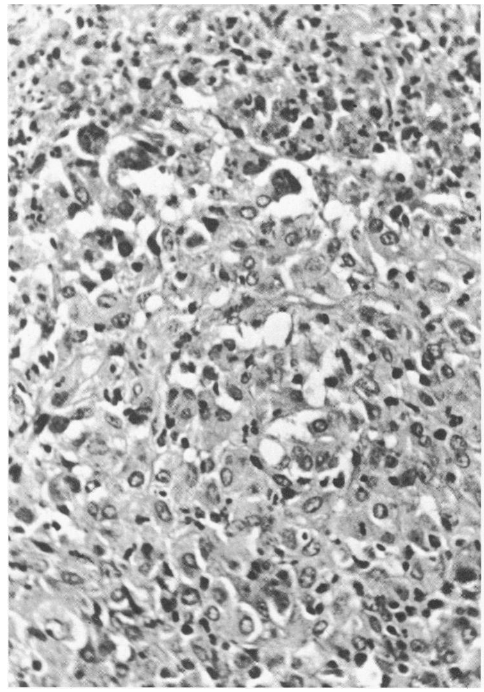

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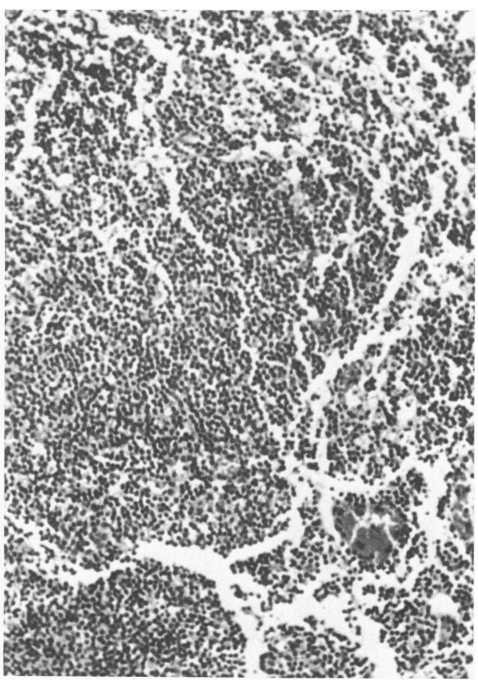

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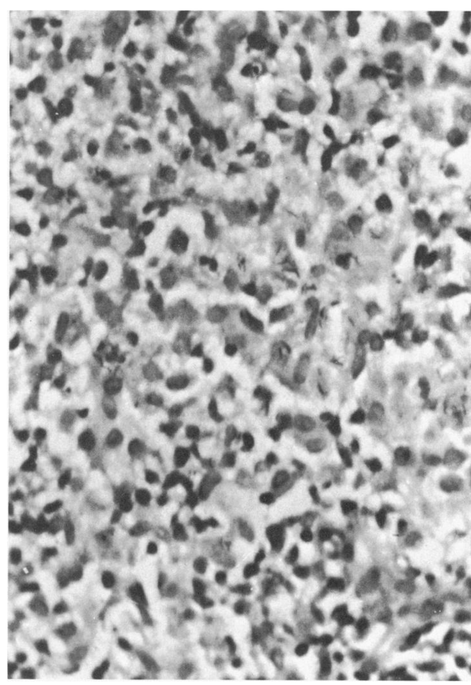

Fig 5—Draining lymph node of the HP animal, 7 days after BCG. A large cluster of macrophages (upper most portion) is seen in the subcapsular region. The paracortical area is cellular and shows numerous mitoses (H & E, × 120). **Fig 6**—Epithelioid cell granuloma with caseation necrosis (uppermost portion) in the draining lymph node of the HP guinea pig, 21 days after BCG (H & E, × 240). **Fig 7**—Draining lymph node of the LP animal, 7 days after BCG. Macrophage accumulation is absent. An involuted lymphoid follicle is seen at the left upper corner. There is moderate cellularity of the paracortical area, but no mitoses are seen (H & E, × 120). **Fig 8**—Diffuse accumulation of macrophages and formation of immature epithelioid cells in the draining lymph node of the LP guinea pig, 21 days after BCG. A fair number of intracellular AFB are seen. Well-formed granulomas and caseation necrosis were absent (Carbolfuchsin and hematoxylin, × 390).