A COMPARATIVE STUDY OF THE REACTION *IN VIVO* AND *IN VITRO* OF RABBIT TISSUES TO INFECTION WITH BOVINE TUBERCLE BACILLI

PART I. OBSERVATIONS ON RABBIT SPLEEN INFECTED *IN VITRO*

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(With Plate 8)

INTRODUCTION

In a previous publication (Fell & Brieger, 1947), it was shown that virulent strains of avian tubercle bacilli which killed rabbits and fowls in 3–4 weeks (Yersin type of infection) did not prevent the active growth of tissue cultures of embryonic chick lung, even when the explants were inoculated with a dense bacillary suspension. Specific lesions and toxic effects were absent, and the cultures survived for 3–6 weeks in spite of heavy intracellular infection. On the other hand, the infection was never eliminated by the tissue. Eventually the living cultures seemed to be disrupted mechanically by the proliferation of the bacilli. These results were confirmed in unpublished experiments in which tissue cultures of young rabbit spleens were infected with virulent avian bacilli.

These observations agreed with those of Smith, Willis & Lewis (1922), who maintained for a few days cultures of embryonic chick tissues infected with moderately virulent organisms of the avian type, but saw no tubercle formation or degenerative changes. Fischer (1927) cultivated tumour tissues of a tuberculous fowl and noted a complete symbiosis between the cells and the bacilli which they contained.

On the other hand, Maximow (1924, 1928), Haagen (1928), and Timofejewsky & Benevolenskaja (1928) found that highly virulent mammalian strains were very injurious to tissue explants from susceptible hosts. These observations suggested that organisms which are pathogenic in the body might be expected to have a rapidly injurious effect on cultures of tissues from a susceptible animal. Our observations on fowl and rabbit tissue cultures infected with bacilli of the avian type, however, have shown that this is not a principle of general application. In view of this disparity between the results described for susceptible tissue cultures infected with avian and mammalian strains respectively, we decided to reinvestigate the behaviour of rabbit tissue cultures infected with virulent bovine tubercle bacilli. The results of this investigation have already been briefly mentioned by one of us (Brieger, 1949), and are described in full in the present paper.

The investigation was made in two parts. In Part I, described in the present

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communication, explants of rabbit spleens were infected after 3 days' growth *in vitro*, and their subsequent history studied. The tissue cultures were infected by placing a droplet of bacillary suspension on the surface of the explant. As this was a somewhat unphysiological mode of infection, in Part II of the research, published in the next paper, the bacilli were introduced into the tissue of the living animal either by direct inoculation into the intact spleen or by intravenous or intrapulmonary injection. The behaviour of the tissue cultures infected in these two ways was compared with that of the spleen and lymph glands left *in situ* in infected rabbits.

MATERIAL AND METHODS

Bacillary strains

Two strains of tubercle bacilli of the bovine type were used in these experiments. For one of these (referred to in the text as BH) we are indebted to Dr Dobson of the Veterinary Laboratory, Weybridge, and for the other (BY) to Dr Wilson, Department of Pathology, Cambridge University. Both strains were derived from the organs of infected cattle and were highly pathogenic to rabbits.

Inoculation of tissue cultures

The tissue was grown by the usual hanging-drop method on large (1 in. square) cover-slips in a mixture of fowl plasma and 11–12-day chick embryo extract. To infect the tissue *in vitro*, explants of normal rabbit spleen were cultivated for 3 days in order to establish an active outgrowth, and were then transplanted to fresh medium. As soon as the medium had clotted, a small loopful of bacterial suspension was deposited on the explant with a fine platinum wire; the culture was mounted, sealed and incubated upside down for 24 hr. to allow the bacilli to settle on the tissue and adjacent clot. The explants were subdivided once or twice and transferred to fresh medium twice a week.

Table 1. *Infection* in vitro

No. of exp.	Strain	No. of cultures examined: Infected	No. of cultures examined: Controls
4	BH	17	14
5	BH: dilution 1	22	20
	dilution 2	19	
7	BY	17	11
11	BH	14	12

Histological technique

About half the tissue cultures were fixed and mounted whole; the rest were serially sectioned (see Table 1). For whole mounts, the cultures were fixed for 5 min. in Zenker's fluid without acetic acid, stained for 5 min. on a hot plate with Ziehl-Neelsen's carbolfuchsin and after being decolorized in the usual way, they were counterstained with Ehrlich's haematoxylin. When they had been differentiated in 5–10 % acetic alcohol, they were 'blued' in running tap water, dehydrated, cleared in xylol and mounted in Canada balsam or Depex.

For sections, the cultures were fixed for 30 min. in 3 % acetic Zenker's fluid, washed and partially dehydrated; to enable the tissue to be orientated in the paraffin wax, the cultures were then stained in a solution of eosin in 95 % alcohol. Next, each cover-slip was placed in a Petri dish containing absolute alcohol, and the clot was scraped away with a cataract knife, so as to leave in the centre of the glass a small rectangular island comprising the explant and the outgrowth; this island was detached with a safety razor blade and transferred to a tube of fresh absolute alcohol. It was cleared in cedar wood oil, embedded in paraffin wax and sectioned in the plane parallel to that of the original cover-slip. This method gave very satisfactory results.

Most of the slides were stained by Ziehl-Neelsen's carbolfuchsin, as described above, and were counterstained with Delafield's haematoxylin.

CULTURES OF NORMAL SPLEEN

When fragments of spleen from a normal rabbit were explanted *in vitro*, lymphocytes, monocytes and macrophages (polyblasts) began to emerge during the first 24 hr. incubation. Putting forth complex pseudopodia, some of the cells crawled through the fibrin clot of the culture medium, while others fell out of the explant to form large masses of round cells in the droplet of fluid which usually appeared beneath the tissue. By the end of the 3rd day each explant was surrounded by a broad outgrowth, though many of the small lymphocytes were dead and being actively phagocyted by the monocytes and macrophages. Larger, less mobile cells of the fibroblastic type were also beginning to migrate from the explant; some were true fibroblasts emerging from the cut ends of blood vessels and sometimes from small pieces of the spleen capsule, but most belonged to the spleen reticulum and contained granules of cell debris and particles of brown pigment.

After the first transfer to fresh medium, the outgrowth was much more profuse and contained a large proportion of reticulum cells. These cells were usually roughly triangular in shape, with long flat processes fanning out into the medium, and very variable in size; they had a large, oval, rather pale nucleus behind which a long spike of cytoplasm projected towards the explant. Other reticulum cells were in the form of macrophages (cf. Maximow & Bloom, 1941); they were more richly branched than the fibroblastic type and the shape of the nucleus was often very irregular, with lobes extending into the cytoplasmic branches. Some reticulum cells assumed a rounded or oval form, often with a relatively large cytocentrum enclosed by a zone of more basophil cytoplasm; the nucleus was usually peripheral. Such cells resembled the 'epithelioid' elements described by Maximow in rabbit tissue cultures infected with B.C.G. (1928) or human tubercle bacilli of low virulence (1924).

Monocytes also were transformed into macrophages but, at least in the early stages of the transformation, they could usually be distinguished from the reticulum cell macrophages by their relatively smaller and more deeply stained nuclei. Especially in the older (9–24-day) cultures, however, it was often impossible to tell whether a given macrophage was derived from a monocyte or a reticulum cell.

After the first 9 days' cultivation the fibroblastic reticulum cells greatly predominated in the outgrowth (Pl. 8, fig. 1), although large and small lymphocytes, *monocytes and macrophages persisted throughout the maximum culture period* (24 days). The lymphocytes and monocytes throve best in the drop of serous fluid which, as described above, often formed beneath the tissue.

Changes within the explants could only be studied in serial sections. In 6-day cultures, the central part of the explant was completely necrotic; it was enclosed by a thick crust of healthy tissue from which cells migrated outwards into the culture medium and inwards into the degenerate mass which they actively phagocyted. As stated above, sections of the controls as well as of the infected cultures were stained with carbolfuchsin. In such preparations many of the granules in the phagocytes were acid-fast, though the red stain was not very intense. All traces of differentiation into red and white pulp usually disappeared during the first 9 days *in vitro*.

In vigorously growing cultures, the necrotic centre had been largely resorbed by the end of the 9th day, and the amorphous remains of the dead tissue were heavily invaded by fibroblastic reticulum cells, macrophages and monocytes. The phagocytes were laden with granular debris and yellow pigment, but contained much less acid fast matter than in younger cultures. Pockets of lymphocytes were scattered throughout the explant. A little undigested fibrin from the surrounding culture medium was sometimes incorporated in the tissue during transplantation; this also was thickly populated by cells.

The interior of the explants changed little during subsequent cultivation, though the number of small lymphocytes usually declined.

CULTURES OF SPLEEN INFECTED *IN VITRO*

Spleen explants from normal rabbits were grown for 3 days and then inoculated with tubercle bacilli of the bovine type (see p. 182). When the cultures were examined 24 hr. later, small masses and groups of bacilli were seen to be distributed over the clot in the neighbourhood of the explant. The originally short rods elongated to several times their previous length and then divided rapidly.

During the first week after inoculation, there was little, if any, difference between the infected cultures and their uninfected controls. In both, the outgrowth consisted mainly of lymphocytes, monocytes and reticulum cells of the fibroblastic, macrophage and epithelioid forms; giant multinucleate cells of the foreign body type, with most of the nuclei congregated in the centre of the cell, were also present in both the infected and control cultures. The epithelioid cells seemed slightly more plentiful in the inoculated cultures. As in the controls, the fibroblastic form of reticulum cell soon predominated over the other cell types in the infected outgrowths.

The migrating monocytes, macrophages and fibroblastic reticulum cells ingested many of the multiplying bacilli, and sometimes macrophages assembled in blackberry-like clusters around the larger bacillary masses. Those organisms which remained free grew vigorously in the medium, but cells near them seemed unaffected and divided normally.

In stained preparations of cultures fixed a few days after inoculation, many cells appeared healthy in spite of the presence of several bacilli in their cytoplasm; some infected cells even underwent mitosis (Pl. 8, fig. 3). Others disintegrated, however, the nucleus became pycnotic and a sheaf of acid-fast filaments sprouted from the cytoplasm. In particular, macrophages clustered round a mass of bacilli, as described above, soon degenerated (cf. Maximow, 1928). The phagocytic cells never became distended with ingested organisms, as did the macrophages of embryonic chick lung cultures infected with tubercle bacilli of the avian type (Fell & Brieger, 1947). Organisms were also present in the multinucleate giant cells.

Since the actively wandering macrophages could tolerate intracellular bacilli for some time, they soon distributed the organisms throughout the explant. Eventually these infected cells died, and the liberated bacilli multiplied freely in the tissue (Pl. 8, fig. 2). Some were taken up by other cells which in turn degenerated, and thus the explant was gradually infiltrated with tubercle bacilli.

Bacillary growth was always most vigorous near the surface of the explant, which by the 10th or 11th day of cultivation was studded with colonies embedded in the tissue but protruding above the surface. It was surprising that such cultures often produced a large, healthy outgrowth with plentiful mitosis (Pl. 8, fig. 4). The cells in immediate contact with the organisms were usually dead, but others nearby seemed quite unharmed. From this it seemed clear that, under the conditions of our experiments, the tubercle bacilli did not produce exotoxins.

Many of the fibroblastic reticulum cells, which at this stage preponderated over other cell types, were quite heavily laden with ingested organisms, yet they showed no obvious signs of degeneration. Even these cells, however, never became so grossly stuffed with bacilli as did the macrophages in tissue cultures infected with the avian type (Fell & Brieger, 1947).

After a heavy inoculation, few cultures survived the 14th day of infection (cf. Maximow, 1928). After this stage the explant was usually almost replaced by a solid mass of organisms and either there was no outgrowth, or only a few scattered cells emerged (Pl. 8, fig. 5). Controls of the same age remained flourishing.

In two cultures fixed and sectioned after 9 and 10 days respectively, there was a curious islet of infected, vacuolated cells very sharply bounded from the surrounding tissue and with fairly normal nuclei. Apart from these rather obscure structures, nothing resembling a tubercle was seen in the cultures at any stage of growth (cf. Maximow, 1928; Haagen, 1928).

In one experiment (Exp. 5, Table 1) the effect of diluting the bacillary suspension was studied (see p. 182). One set of spleen cultures was infected with a suspension of medium concentration, another with the same suspension diluted 1 : 100 and a third (control) set was not inoculated. During the first 14 days the three series grew equally well, although colonies of bacilli could be seen in the interior of some cultures in both the infected groups. By the 17th day, however, infection had greatly increased in the cultures which had received the more concentrated suspension, and some were growing feebly; those treated with the diluted suspension were still vigorous, though obviously containing relatively large masses

of bacilli. The experiment was terminated 21 days after inoculation, by which time *most of the cultures had been fixed; some of the survivors in each infected series* were still growing surprisingly well, in spite of dense bacterial infiltration. The controls were normal and active.

In heavily infected spleen cultures, the bacterial colonies appeared strongly acid-fast, but close inspection revealed a high proportion of non acid-fast elements mixed with and partly masked by the red stained organisms.

DISCUSSION

The results we have described confirm the observations of Maximow (1928) that in 10–20 days virulent bovine bacilli disrupt and destroy cultures of rabbit tissue. Maximow thought that the bacilli had an exotoxic action on tissue cultures from a susceptible species. Haagen (1928) and Timofejewsky & Benevolenskaja (1928) gave a similar interpretation of their results, but we have found no evidence to support this view. As recorded above, apparently healthy cells, many of them in mitosis, were often seen close to ropes of virulent bacilli, and sometimes a densely infected explant was surrounded by an active zone of outgrowth. In this respect our results agree with what is observed during the early stages in the experimental infection of animals (Woodruff, Kelly & Leaming, 1942).

Phagocytosis by macrophages is a constant feature of all experimental infection both *in vivo* and *in vitro*. Maximow thought that in rabbit tissue cultures the cells were attracted by bacilli of the human type (1924), which are not very pathogenic for the rabbit, but not by virulent bovine organisms, which were much less readily phagocyted (1928). As described above, in rabbit spleen cultures virulent bovine bacilli were ingested quite readily, but not nearly to the same extent as avian tubercle bacilli.

In our experiments, ingestion was followed by a period, brief for the bovine and much longer for the avian bacillus (Fell & Brieger, 1947) of symbiosis between the host cell and the parasite; cells even completed mitosis in spite of the presence of several virulent bovine bacilli in their cytoplasm. These observations agree with those of Lurie (1942), who showed that when tissue infected *in vivo* with bovine bacilli was transferred to the anterior chamber of the eye, this environment permitted the bacilli to grow within the macrophages, 'while the cells appeared well preserved and alive'.

These results suggest that the living bovine bacilli do not have an endotoxic effect, and the question arises as to how they eventually kill the host cells. It is possible that their habit of growth may be one, but probably not the only, destructive factor. In our previous paper (Fell & Brieger, 1947), it was shown that the macrophages of susceptible tissue in culture could contain an enormous number of virulent avian bacilli without disintegrating; the organisms were pressed together in large round vacuoles which enormously distended the cytoplasm, but did not rupture the cell membrane. On the other hand, the ingested bovine bacilli tended to grow in spiky skeins which perhaps penetrated the cell membrane more readily than the more discrete avian bacilli which could be tidily packed into vacuoles. A similar difference between the reaction of macrophages to

avian and bovine organisms has been observed in the chick embryo (Costil & Bloch, 1938; Canat & Opie, 1943; Moore, 1942). It is also possible that the growing bovine bacilli may utilize materials in the host cytoplasm which are needed by the cell, which thus may be gradually starved by the parasites.

It is interesting that the epithelioid cells and giant cells which form so readily in rabbit spleen cultures do not seem to destroy ingested bovine bacilli *in vitro*, or even to arrest their multiplication. This was also observed by Rich (1946).

No tubercles were formed in our tissue cultures in response to a virulent bovine tuberculous infection, although there seemed to be more macrophages of the epithelioid type than in the uninfected controls. Maximow (1928) and Timofejewsky & Benevolenskaja (1928) explained the absence of tubercles in their tissue cultures infected with virulent mammalian strains by the rapidity of the necrotic process. In our experiments, however, the tissue was not always destroyed so quickly, and there was time for tubercles to have formed.

The explanation of this almost complete lack in tissue cultures of the characteristic lesions of tuberculosis probably lies in the simplification of physiological and morphological conditions which cultivation *in vitro* involves. There are important histological differences between the same tissue *in vivo* and *in vitro*; in the explants there is no vascular or nervous system, polynuclear cells are absent and lymphocytes, though usually present in rabbit spleen explants throughout the maximum culture period, are fewer than *in vivo*. Certain types of serological reaction are eliminated in culture.

Moreover, it is unlikely that allergy develops in tissue cultures. According to modern views, allergy is held to be responsible for the destructive action of the bacilli on the tissues of the intact animal and is thought to mobilize forces in the host cell which terminate the state of symbiosis and cause the destruction of the bacilli and the liberation of their endotoxins. This question will be discussed further in Part II.

SUMMARY

1. The effect of virulent bovine tubercle bacilli on rabbit tissue *in vitro* was studied.

2. When normal spleen cultures were infected *in vitro* the cells actively phagocyted the bacilli. At first the phagocytes tolerated the organisms, and in the course of their wanderings spread the infection throughout the tissue. Some infected cells even underwent mitotic division.

3. Eventually the infected cells broke down, and the freed bacilli continued to grow in the tissue. Fourteen days after infection, outgrowth had usually ceased and the explants were almost completely replaced by bacilli.

4. Actively growing spleen cultures were unable to suppress even a slight infection with virulent bovine bacilli.

5. No tubercles were formed in the cultures.

6. There were no indications of an exotoxic action of the bacilli on the cells in tissue culture, and densely infiltrated explants were often surrounded by a large zone of outgrowth.

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EXPLANATION OF PLATE 8

Photographs by Mr V. C. Norfield

Note. All the preparations were stained with Ziehl-Neelsen's carbolfuchsin and haematoxylin.

Fig. 1. Uninfected (control) culture of spleen (Exp. 5, Table 1) after 17 days' growth. Fibroblastic reticulum cells predominate in the outgrowth, but other cell types, including macrophages and small lymphocytes, are present. (Whole mount, × 32.)

Fig. 2. Spleen culture (Exp. 4, Table 1), 9 days after infection *in vitro*. A group of infected cells have broken down and the freed bacilli are multiplying in the tissue. (Section, × 580.)

Fig. 3*a*, *b*. Two reticulum cells undergoing normal mitosis (metaphase) in spite of the presence of virulent bovine tubercle bacilli in their cytoplasm. From a culture fixed 10 days after infection *in vitro* (Exp. 5, Table 1). (Section, × 1300.)

Fig. 4. Spleen culture after 11 days' infection *in vitro* (Exp. 11, Table 1). The explant is densely infiltrated with bovine tubercle bacilli, but the culture is still growing actively. (Whole mount, × 32.)

Fig. 5. Spleen culture after 19 days' infection *in vitro* (Exp. 4, Table 1). Outgrowth has ceased and the tissue is almost completely replaced by acid-fast bacilli. (Section, × 71.)

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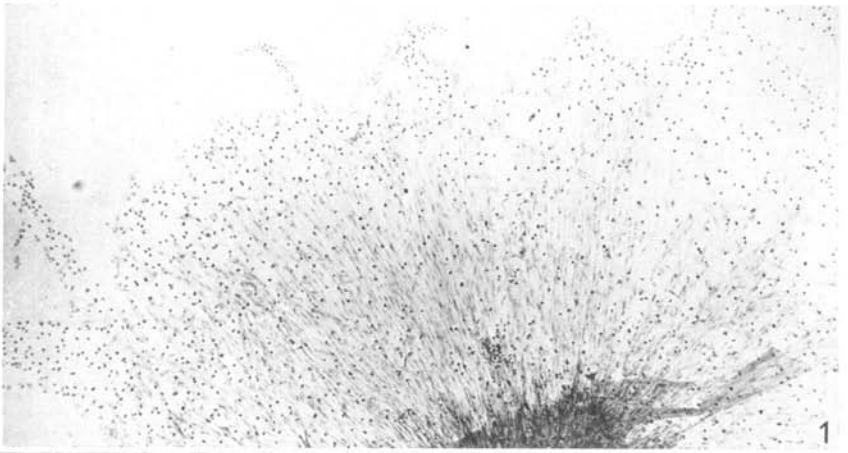
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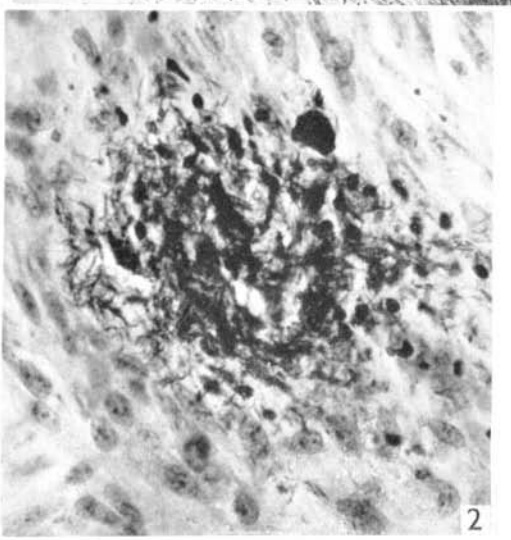
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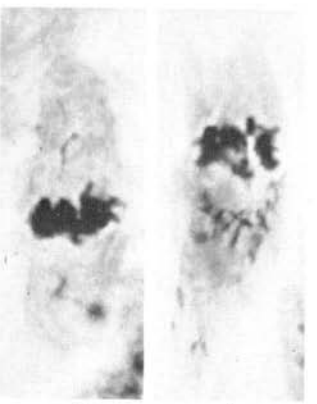
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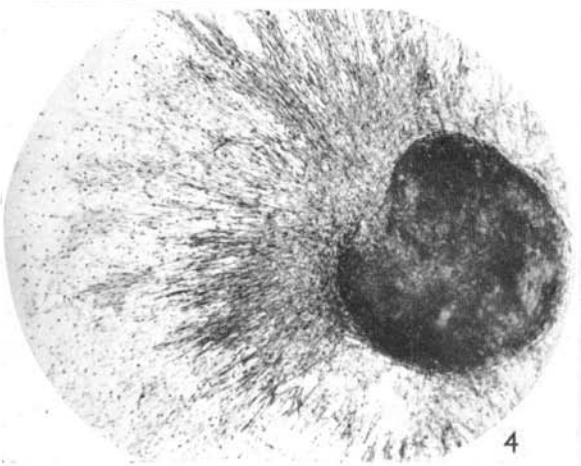
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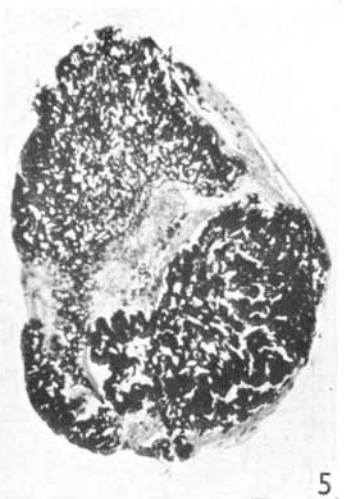
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