Adjuvant arthritis in the rat

Distribution of fluorescent material after footpad injection of rhodamine-labelled tubercle bacilli

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Vernon-Roberts, B., Liyanage, S. P., and Currey, H. L. F. (1976). *Annals of the Rheumatic Diseases*, 35, 389–397. Adjuvant arthritis in the rat. Distribution of fluorescent material after footpad injection of rhodamine-labelled bacilli. Adjuvant disease in the rat may represent a cell-mediated response to tuberculous material disseminated from the original injection site, but previous studies have provided only indirect evidence for this dissemination. In the present experiments tubercle bacilli labelled *in vitro* with rhodamine isothiocyanate (RITC) were injected into a footpad as Freund's complete adjuvant. Serial studies showed two varieties of fluorescent material in the tissues (1) intracellular and extracellular intact and fragmented bacilli, and (2) amorphous intracellular material. Both types of material were identified in the injected foot and draining lymph nodes. Bacilli were also identified in the contralateral knee joint, peritoneum, pleura, lung, and liver, while amorphous material alone appeared in the spleen. The presence of intact bacilli was confirmed by positive Ziehl-Nielsen staining of the organisms, but the amorphous intracellular material did not stain positively by this method.

The use of RITC-labelled organisms considerably reduced the severity of adjuvant disease. Most of the organisms identified in sites distant from the injected limb were not situated within foci of inflammation. Marked differences in processing of the tuberculous material (and lack of dissemination of intact bacilli) were noted when labelled organisms were injected in saline instead of in oil.

Adjuvant arthritis can be induced in the rat by a single intradermal injection of Freund's complete adjuvant (dead tubercle bacilli in oil). It has been postulated that the widespread polyarthritis and other lesions which appear 10 to 15 days after this injection represent a cell-mediated reaction to tuberculous material reaching the joints and other tissues from the original injection site (Waksman, Pearson, and Sharp, 1960; Quagliata and Phillips-Quagliata, 1972).

In order to produce this arthritis it is necessary to satisfy fairly precise experimental conditions, including the constitution of the adjuvant, route of injection, species and strain of rat, etc. Such variables might in theory act by influencing either the immune response of the rat or the dissemination of tuberculous material in the tissues. In a previous

study (Liyanage, Currey, and Vernon-Roberts, 1975) we have shown that the production of arthritis requires tuberculous aggregates of less than 90 μm in size, and that this size requirement probably operates by influencing the resulting cell-mediated immune response.

The factors influencing the distribution of tuberculous material to distant sites are unknown, and indeed the evidence for dissemination of tuberculous material from the injection site is only indirect (Quagliata and Phillips-Quagliata, 1972), previous tracing experiments having given somewhat inconclusive results (Jones and Ward, 1962; 1964). We have therefore investigated the distribution of fluorescent material in rats after an injection of a suspension in oil of dead tubercle bacilli labelled *in vitro* with rhodamine isothiocyanate (RITC).

Materials and methods

PREPARATION OF LABELLED TUBERCLE BACILLI IN ADJUVANT

Initially tubercle bacilli were labelled with fluorescein isothiocyanate (FITC). This produced strong labelling of the organisms. However, it was found that necrotic tissue and phagocytosed intracellular debris in the injected footpad and its draining lymph nodes gave rise to much nonspecific green autofluorescence when the tissues were being examined for specific FITC fluorescence (which is also green). This problem proved impossible to overcome with special excitation and barrier filters. However, the use of RITC circumvented this problem since autofluorescence did not occur to any significant degree at the wavelength used for specific RITC excitation.

Aggregates of dried, heat-killed *Mycobacterium tuberculosis*, mixed strains C, DT, and PN (Central Veterinary Laboratory, Weybridge) were prepared in the size range 45–63 μm using metal sieves as previously described (Liyanage and others, 1975). The tubercle bacilli were then labelled with RITC by a modification of the method of Rickles and others (1969). Aggregates of tubercle bacilli were suspended in glycine buffer at pH 9.0 and RITC was added to a final concentration of

50 mg/ml. After stirring for 60 minutes at room temperature with a magnetic stirrer, the aggregates were washed three times with glycine buffer to remove unbound RITC. Washed aggregates were then suspended in heavy mineral oil as Freund's complete adjuvant (FCA) at a final concentration of 6 mg/ml.

ARTHRITIS

Sprague-Dawley rats weighing 200–250 g were used throughout, and arthritis was induced by an intradermal injection of 0.05 ml FCA into the right hindfoot pad. Control animals were given unlabelled aggregates incorporated in FCA, RITC-labelled aggregates suspended in normal saline, or RITC mixed with heavy mineral oil.

EXAMINATION OF TISSUES

Rats were killed at various intervals after footpad injection. Cryostat sections 8 μm thick, of unfixed tissues were made of the injected footpad, iliac lymph nodes draining the injected leg, synovial tissues from the contralateral knee joint, lung, spleen, and liver; and smears were made of thoracic duct lymph and peripheral blood. These specimens were examined for the presence of fluorescent material by epifluorescence using a Zeiss binocular photomicroscope having an HBO-200 mercury

FIG. 1 (a) Low-power view of rhodamine-labelled 45–63 μm aggregates of tubercle bacilli suspended in heavy mineral oil before footpad injection, showing the general uniformity of size of the aggregates. $\times 60$. (b) High-power view of free (i.e. not forming part of the aggregates seen in (a)) rhodamine-labelled tubercle bacilli in the mineral oil before footpad injection, showing that both intact and fragmented bacilli are present. $\times 1000$

vapour light source and equipped with the III RS condenser containing sets of the appropriate exciter filters, reflectors, and barrier filters for selective RITC excitation and observation. The morphology of cells and tissues within each field was examined by changing from epillumination with ultraviolet light to phase-contrast examination by transmitted tungsten illumination. Sections were also stained by the Ziehl-Nielsen (ZN) technique for examination by conventional light microscopy. Specimens of injected footpad and synovial tissues from the contralateral knee joint were fixed in 2.5% phosphate buffered glutaraldehyde at 4°C and processed for electron microscopy.

Results

LABELLING OF TUBERCLE BACILLI WITH FITC AND RITC

Using the labelling technique described, all the aggregated and free intact tubercle bacilli and bacilliary fragments incorporated in FCA were labelled and fluoresced strongly (Fig. 1*a, b*). There

appeared to be no unlabelled bacilli or fragments. The label could not be removed *in vitro* by repeated washings with buffer, or treatment with 0.1 N HCl, 0.1 N NaOH, ethanol, acetone, chloroform, ethyl acetate, or trypsin (Rickles and others, 1969).

DISTRIBUTION OF FLUORESCENT MATERIAL OF RATS INJECTED WITH RITC-LABELLED TUBERCLE BACILLI INCORPORATED IN FCA

Two varieties of fluorescent material were seen in the tissues of rats injected with RITC-labelled tubercle bacilli incorporated in FCA; they were (1) intracellular and extracellular intact and fragmented tubercle bacilli (Figs. 3–6) and (2) amorphous mottled intracellular material (Fig. 7). The results are summarized in Table I.

Injected footpad

Abundant bacilli persisted in the injected footpad up to 28 days after injection (Fig. 2*a, b*). Intact and fragmented bacilli were contained in the aggregates

FIG. 2 (a) Medium-power view of injected footpad on day 3, showing large aggregate in centre of field surrounded by inflammatory cells containing phagocytosed tubercle bacilli, and several smaller aggregates nearby. $\times 200$. (b) Low-power view of injected footpad on day 21, showing persisting aggregate at the top of the field, many oil droplets with tubercle bacilli at their periphery (arrows), and abundant intracellular labelled material. $\times 30$

Table I Amounts of labelled material present in tissues at various intervals after injection into the rat footpad of rhodamine-labelled tubercle bacilli incorporated in Freund's complete adjuvant

Days after footpad injection	Injected footpad	Draining lymph node	Thoracic duct lymph	Contralateral knee joint	Peritoneum and pleura	Spleen	Liver	Lung
3	+	+	—	+	+	+	+	+
5	+	+	—	+	+	+	+	+
7	+	+	—	+	+	+	+	+
8	+	+	—	+	+	+	—	+
10	+	+	—	+	+	+	+	+
14	+	+	—	+	+	+	+	+
21	+	+	—	+	+	+	+	+
28	+	+	—	+	+	+	—	+

B = intact and/or fragmented bacilli; M = intracellular mottled amorphous material; — = none; + = occasional; ++ = sparse; +++ = abundant; ++++ = very abundant.

Table II Amounts of labelled material present in tissues at various intervals after injection into the rat footpad of rhodamine-labelled tubercle bacilli suspended in normal saline

Days after footpad injection	Injected footpad	Draining lymph node	Contralateral knee joint	Peritoneum and pleura	Spleen	Liver	Lung
5	+	+	—	—	+	—	—
8	+	+	—	—	+	—	—
14	+	+	—	—	+	—	—
21	+	+	—	—	+	—	—

B = intact and/or fragmented bacilli; M = intracellular amorphous material; — = none; + = occasional; ++ = sparse; +++ = abundant; ++++ = very abundant.

FIG. 5 *Skeletal muscle containing tubercle bacilli within lymphatic channels draining the injected footpad on day 3.* $\times 500$

and in aggregate-free oil droplets which slowly but progressively diminished in size. Aggregates were surrounded by an inner zone of neutrophil polymorphs which contained small amounts of labelled material, and an outer zone of macrophages which always contained large amounts of labelled material and some intact bacilli. Electron microscopy showed that the majority of the polymorphs were degenerate, and that the outer zone of macrophages possessed long processes extending between the polymorphs towards the tuberculous material in the centre of these lesions.

Oil droplets were initially also surrounded by polymorphs, but these were soon replaced by macrophages alone which contained labelled material and intact bacilli; the oil droplets rapidly became depleted of labelled bacilli, and electron microscopy showed that the oil had become enclosed within a

unit membrane structure separating the oil from the surrounding macrophages. In the tissues between the aggregates and oil droplets, there were extensive sheets of macrophages containing abundant labelled material and occasional intact or fragmented bacilli. Intracellular and free intact and fragmented bacilli were present within lymphatics draining the footpad (Fig. 3) throughout the period of examination.

Draining lymph nodes

Increasing amounts of labelled material were present in the internal iliac nodes draining the injected leg. Up to day 5 the material, predominantly in the form of free intact and fragmented bacilli, was situated in the afferent lymphatics and subcapsular sinuses (Fig. 4). After day 5 oil droplets containing bacilli were frequently seen in the paracortical areas, with macrophages around the oil droplets containing intact and fragmented bacilli. After day 7 the macrophages lining sinuses and within paracortical areas contained moderate amounts of amorphous intracellular mottled material.

Thoracic duct lymph

No free or intracellular labelled material was seen in samples of thoracic duct lymph obtained by short-term cannulation. The examination of samples obtained by long-term thoracic duct drainage will be the subject of future studies.

Contralateral knee joints

Small numbers of intact or fragmented bacilli were present within, and on the surface of, synovial lining cells (Fig. 5a, 5b) throughout the 28 days of the study. Labelled material was absent from the deeper connective tissues, and was not seen within the inflammatory cells infiltrating the joint when active synovitis was present. There was no apparent difference between the amount of labelled material in synovial lining cells of joints exhibiting active synovitis and those which were not inflamed.

FIG. 4 *Numerous bacilli within afferent lymphatics and distended subcapsular sinuses of a lymph node draining the injected leg on day 5.* $\times 500$

FIG. 5 (a) Synovial tissue of knee joint of noninjected leg on day 7, showing bacilli within synovial lining cells. $\times 320$

FIG. 5 (b) Synovial tissue of knee joint of noninjected leg on day 14. Shows bacilli within synovial lining cells. $\times 320$

Peritoneum and pleura

Small numbers of intact and fragmented bacilli were present within, and on the surface of, the lining cells of the pleura (Fig. 6) and peritoneum throughout the period of the study.

FIG. 6 Pleura on day 3, showing tubercle bacilli within and adherent to pleural mesothelial cells. $\times 305$

FIG. 8 Lung on day 3, showing discrete and clumped tubercle bacilli within macrophages in parenchyma and free bacilli within bronchioles. $\times 500$

FIG. 7 Spleen on day 5, showing abundant intracellular amorphous mottled material within macrophages of the marginal zones, and absence of intact or fragmented bacilli. $\times 340$

Spleen

No intact bacilli or bacilliary fragments were seen in the spleen, apart from one animal in which a few fragments were seen in macrophages of the marginal zone at the periphery of the lymphoid tissue of the white pulp on day 10. However, abundant amorphous mottled labelled material was seen within macrophages of the marginal zone and the red pulp throughout the 28 days of the study (Fig. 7).

Liver

Very small numbers of bacilliary fragments were occasionally seen within Kupffer cells of the liver between day 3 and day 14.

Lung

Large numbers of intracellular and intra-alveolar intact and fragmented bacilli were present in the parenchyma of the lung (Fig. 8), particularly in the periphery, and tuberculous granulomata were present after day 7.

FIG. 9 Section of synovial tissue of knee joint of noninjected leg on day 14 stained by Ziehl-Nielsen technique, showing intact and fragmented alcohol and acid-fast tubercle bacilli within synovial lining cells. $\times 1000$

DISTRIBUTION OF ZIEHL-NIELSEN POSITIVE MATERIAL IN RATS INJECTED WITH RITC-LABELLED TUBERCLE BACILLI INCORPORATED IN FCA

The presence of intact or fragmented bacilli was confirmed by positive ZN staining of *Mycobacteria* in the injected footpad, draining lymph nodes, contralateral knee joint (Fig. 9), peritoneum, pleura, and lung; although the numbers of bacilli or fragments showing positive alcohol- and acid-fast staining typical of *Mycobacteria* were markedly less than the numbers visible by fluorescent examination of the same tissues. The mottled amorphous intracellular fluorescent material seen in the injected footpad, draining lymph nodes, and the spleen did not exhibit a positive staining reaction using the Ziehl-Nielsen technique.

DISTRIBUTION OF FLUORESCENT MATERIAL IN RATS INJECTED WITH RITC-LABELLED TUBERCLE BACILLI SUSPENDED IN NORMAL SALINE

While abundant fluorescent material persisted in the injected footpad throughout the 21 days of this part of the study, after day 5 the amount of amorphous intracellular material greatly exceeded the amount of intact and fragmented bacilliary material; this was the reverse of the findings in the animals which had been injected with bacilli suspended in FCA. No other tissues apart from the footpad contained intact or fragmented bacilli. However, while the draining lymph nodes and spleen contained much amorphous intracellular material up to day 21, the knee joint, peritoneum, pleura, liver, and lung were devoid of labelled material throughout. The results are summarized in Table II.

DISTRIBUTION OF LABELLED MATERIAL IN RATS INJECTED WITH RITC MIXED WITH HEAVY MINERAL OIL

Fluorescence was restricted to oil droplets in the injected footpad, and no labelled material was seen in any of the other tissues.

DEVELOPMENT OF ADJUVANT ARTHRITIS IN RATS INJECTED WITH RITC-LABELLED TUBERCLE BACILLI INCORPORATED IN FCA

Rats injected with RITC-labelled tubercle bacilli in FCA developed less severe arthritis at a later stage with fewer secondary lesions when compared with our previous findings (Liyanage and others, 1975) using unlabelled bacilli of the same strains in the same breed of rats.

Discussion

The results establish that injection into the rat footpad of Freund's complete adjuvant containing rhodamine-labelled tubercle bacilli resulted in rapid and widespread dissemination of fluorescent material. Some of this material was in the form of actual bacilli (intact or fragmented) and, guided by the distribution of these fluorescent organisms, it was possible to confirm the presence of *M. tuberculosis* by ZN staining. Particularly significant was the identification of intact and fragmented tubercle bacilli in the synovial lining cells of joints distant from the injection site. This dissemination, for which the work of Quagliata and Phillips-Quagliata (1972) provided strong indirect evidence, has not been clearly established previously, although Jones and Ward (1962, 1964) were able to show some radioactivity in the tissues on the extremities after intradermal tail injection of Freund's complete adjuvant containing *M. butyricum* biosynthetically labelled with ^{14}C or ^3H . Using Ziehl-Nielsen staining and ^{125}I -labelling of organisms, Berry, Willoughby, and Giroud (1973) were unable to show dissemination of organisms in rats with adjuvant arthritis; while in the rabbit, Doble and others (1975) found no evidence that ^{125}I -labelled *M. tuberculosis* travelled to distant joints after subcutaneous injection of Freund's complete adjuvant.

Rhodamine-labelled material also appeared as amorphous and mottled fluorescence within cells in

the injected footpad, the draining lymph nodes, and the spleen. This form of the material did not give a positive staining reaction with the ZN method, and its exact nature has not been established. The time course of its appearance and distribution suggests that it derived from the degradation of labelled organisms phagocytosed by macrophages in the injected foot or draining lymph node; its presence in the spleen could be due to the rapid degradation of circulating bacilli in that organ, or result from digested material being carried intracellularly in macrophages to the spleen.

The experiments in which the RITC-labelled organisms were injected into the footpad in a saline suspension instead of in oil were highly informative. In this case intact and fragmented organisms were seen only in the injected footpad; disseminated material consisted exclusively of amorphous material in the lymph nodes and spleen. The local appearance in the injected foot suggested much more rapid processing of Mycobacteria to the intracellular amorphous material compared with the situation when oil was included in the injection. Retardation of processing by macrophages might be the essential role which the oil plays in the production of adjuvant disease. It may also be important in the conventional adjuvanticity of Freund's complete adjuvant.

The distribution of the fluorescence observed in these experiments suggests that, after footpad injection of Freund's complete adjuvant (which contains aggregated and free organisms), the tuberculous material is disseminated both as intact (complete or fragmented) bacilli, either free or within macrophages, and also as processed amorphous material within macrophages. Our electron microscope studies indicated that the breaking down of the aggregates in the footpad is predominantly brought about by macrophages, with polymorphs playing a lesser role. In this context our earlier findings (Liyanage and others, 1975) regarding the need for aggregates of smaller size in order to produce arthritis may be relevant. Conceivably, it may even be the presence of a relatively larger proportion of free (nonaggregated) organisms in this 'small-aggregate' material which makes it arthritogenic.

The distribution of fluorescent material within the injected limb indicates that injected tuberculous material, both free and intracellular, travels from the footpad to the draining lymph nodes via the lymphatics. It is difficult to conceive that its onward passage to wider distribution could be other than via the thoracic duct and blood stream. Our failure to observe fluorescent material in either thoracic duct lymph or blood probably reflects how relatively sparse this 'in transit' material is in these fluids. Extirpation experiments by others suggest that the

essential role of the footpad injection site is very transient (Jones and Ward, 1962), while that of the regional lymph nodes continues for about 5 days after injection (Newbould, 1964).

It was notable in these experiments that rats injected with RITC-labelled *M. tuberculosis* in Freund's complete adjuvant developed much less severe arthritis than animals receiving unlabelled bacilli. The reason for this is not clear, but it seems possible that the RITC may combine with sites on the wax D fraction of the mycobacterial cell wall in a manner similar to that which occurs with proteins (Shinozaki, 1960, quoted by Pearson and Wood, 1969) and thus reduce the arthritogenicity of the wax, possibly in a manner comparable to that which Pearson and Wood (1969) have postulated for foreign proteins mixed with the adjuvant.

There clearly remain certain problems in reconciling these results with the hypothesis that adjuvant disease represents a cell-mediated response to tuberculous material disseminated from the original injection site. The first is the fact that organisms were not more numerous in joints showing evidence of inflammation, and indeed most of the organisms identified in sites remote from the injected foot appeared not to be related to any inflammatory process. The second problem is that of reconciling the continued presence (for at least a month) of tuberculous material disseminated through the tissues with a self-limiting disease in which the signs of inflammation are subsiding towards the end of this period.

The precise interpretation of the experiments described here is further complicated by the heterogeneity of the tuberculous material injected and uncertainty regarding the relationship of positive ZN-staining to RITC-staining, and the relationship of both to the postulated antigenic moiety of the organism in this model. Electron microscopy of the tubercle bacilli showed that many of the organisms were abnormal even before labelling with RITC, having lost cell wall material or being altered in other ways. Stained with RITC, the fluorescence of individual organisms was variable; while the majority were heavily and uniformly labelled, many had a 'beaded' appearance owing to patchy or punctate labelling, and a few were barely visible under identical conditions of examination. After footpad injection of RITC-labelled organisms in Freund's complete adjuvant, considerably greater numbers of organisms could be identified in the knee joint of the noninjected leg by fluorescence than by ZN staining: this discrepancy could be the result of a difference in the sensitivity of the two staining methods, a difference in the moiety of the organism stained, or because prior labelling with RITC interferes with subsequent staining by the ZN technique.

Because the use of RITC-labelled organisms markedly reduces the severity of adjuvant disease, the observation that RITC-labelled bacilli were present in both inflamed and noninflamed joints might be explained (in terms of the hypothesis mentioned above) in one of the following ways. (1) That moiety of the bacillus labelled by RITC or ZN staining is not concerned in the genesis of adjuvant disease; (2) the presence of diffuse RITC-labelling itself prevents organisms from initiating an inflammatory reaction; (3) any bacilli initiating an inflammatory response are rapidly degraded by inflammatory cells; or, (4) labelled and unlabelled organisms have different tissue distributions. The

fact that antigen appears to persist at a time when the inflammatory reaction is waning may be a reflection of the fact that a critical state of immune reactivity (perhaps a balance between cell mediated and humoral antibody levels) may be essential for the expression of this disease, and that these conditions obtain only for a limited period. If so, the limiting factor might be the immune status of the animal, not the continued presence of antigen.

We are grateful to Dr. I. Ginsburg for helpful advice; to Mr. G. H. Bowden for studying the binding of FITC and RITC to tubercle bacilli; and to the Arthritis and Rheumatism Council for financial support.

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