An experimental animal model of granulomatous bowel disease

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SUMMARY A study has been undertaken of the granulomatous response induced in the ascending colon and terminal ileum of the guinea pig by the direct inoculation of mycobacterial antigens. Live BCG (Pasteur) 2×10^7 at two weeks induced epithelioid cell granulomas in both large and small bowel and in the draining lymph nodes. The area of infiltration was significantly greater for a given inoculum in the large bowel. Acid fast bacilli were present on Ziehl Neelson stained sections of the large bowel infiltrate, but only rarely in sections from the small bowel lesions. The response to skin testing with a standardised amount of purified protein derivative was less in animals inoculated in the small bowel. Inoculation with 2×10^9 cobalt irradiated BCG gave rise, at five weeks, to granulomas containing lesser numbers of epithelioid cells and caseation was sometimes evident. There was a similar but smaller difference in the degree of infiltration at the two inoculation sites. Ziehl Neelson staining failed to reveal the presence of acid fast bacilli in any sections of the bowel infiltrates. Skin testing with purified protein derivative gave a response which was greater in animals inoculated in the small bowel. An identical dose of Cobalt irradiated *M leprae* induced at five weeks a predominantly macrophage granuloma in both the large and small bowel, with no significant difference in the degree of infiltration at the two sites. No acid fast bacilli were seen in Ziehl Neelson stained sections of the bowel and skin testing with purified protein derivative was reduced. These findings and their relevance to studies of the aetiology of Crohn's disease are discussed.

There have been many attempts to produce an animal model of inflammatory bowel disease (IBD) using a variety of mineral or biological reagents.¹⁻³ Work in the 1970s⁴ using homogenates of human Crohn's disease tissue inoculated into the ileum of rabbits showed, over a prolonged period in some cases, the development of granulomatous changes in the bowel wall. Subsequent efforts to repeat this work have met with mixed success.^{5,6}

More recently⁷ an organism belonging to the Runyon group 111 mycobacteria, perhaps most closely related to *Mycobacterium paratuberculosis* was isolated after prolonged culture from resected bowel tissue of a patient with Crohn's disease. Other workers have described the isolation of a variety of

cell wall deficient forms of mycobacteria from tissue of patients with Crohn's disease.^{8,9} No information is, however, available on the effect of the direct inoculation of mycobacteria into the bowel wall. Thus it was considered of interest to ascertain these responses and to compare them with those known to result from the inoculation of mycobacteria into the peripheral tissues of animals of the same strain and sex.^{10,11} Two different mycobacteria were used: BCG which produced a predominantly epithelioid cell granuloma (Fig. 1) and *Mycobacterium leprae* which in the guinea pig produced a predominantly macrophage type granuloma.¹⁰

Methods

ANIMALS

Outbred Hartley strain female guinea pigs weighing 280-340 g were used. They were fed on an RGP pelleted diet supplemented with cabbage.

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Fig. 1 Granulomatous infiltration of terminal ileum two weeks after inoculation with live BCG (Pasteur). Haematoxylin and eosin. $\times 70$.

MYCOBACTERIA

Live BCG was of Pasteur strain obtained by courtesy of the Institut Pasteur, Paris. Cobalt irradiated armadillo derived *Mycobacterium leprae* and cobalt irradiation of BCG (Pasteur), was provided courtesy of Dr R J W Rees, National Institute for Medical Research, Mill Hill, London. The mycobacteria were obtained as a suspension in saline. The BCG organisms were counted by the method of Miles and Misra¹² for viable organisms. *M. Leprae* were counted by the method of Hart and Rees¹³ which gives a count of the total number of intact organisms. Live *M. Leprae* was not used because of legal restrictions owing to its pathogenicity in man.

LAPAROTOMY

Guinea pigs were anaesthetised with intramuscular neuroleptanalgesia using a combination of Hypnorm (Janssen Pharmaceuticals Ltd) and Hypnoval (Roche Products Ltd). The abdomen was shaved and the skin cleansed with 70% alcohol. A sterile drape with a central aperture was secured in position with an Opsite dressing (Smith & Nephew Medical Ltd). A 3 cm lower abdominal midline incision was made to

the peritoneum which was lifted free of the abdominal contents with non-toothed forceps and incised. The edges of the wound were grasped with Poirers tissue forceps and reflected. This approach gave ready access to the inoculation sites in the terminal ileum and the ascending colon. After inoculation (see below) the bowel was returned to the abdomen and the muscle layer closed carefully with a continuous 3:0 Vicryl suture (Ethicon Ltd, UK). The skin was closed with 4:0 interrupted Vicryl sutures. The sutures were reinforced using Histoacryl Tissue Adhesive (B Braun Melsungen AG, West Germany).

INOCULATION

All inoculations were of 50 μ l and standard doses of organisms (2×10^7 BCG, 2×10^6 irradiated BCG, and 2×10^6 irradiated *M. leprae*) were used. Inoculation was performed, using a Hamilton microlitre syringe and a 32G needle, into the serosa of the antemesenteric wall of the bowel at the level of either the most distal terminal ileal Peyer's patch or the most proximal Peyer's patch of the ascending colon. The doses used were based upon earlier work^{10,11} which had produced a satisfactory response in the posterior

auricular lymph node of the guinea pig, maximal at two and five weeks respectively. They have also been shown to induce sensitivity to 25 µg Purified Protein Derivative (PPD) of tuberculin on skin testing.^{10 11 14} This dose produces no response in unsensitised guinea pigs.¹⁵

CONTROLS

Control animals were inoculated identically with the same volume of physiological saline.

SKIN TESTS

Twenty four hours before harvesting the right flank of each animal was shaved and an injection of 25 µg dialysed purified protein derivative in a volume of 0.1 ml was injected intradermally. The delayed hypersensitivity reaction was read at 24 hours using Schnelltaster Kröplin callipers A 02T to measure the increase in skinfold thickness. The results are expressed as specific increases in skin thickness, which represent the reading (10-imm) at the skin test site, minus the average thickness of the normal skin on both sides of the site.

HARVESTING

After terminal anaesthesia the abdomen was reopened through the original incision. After a general examination of the abdominal contents, the inoculated area of bowel was excised, trimmed and placed in 10% formal saline as were representative samples of liver and spleen. The ileocolic and caecal lymph nodes, which drain primarily the ascending colon and terminal ileum respectively, were excised, weighed and prepared for fixation in Carnoy's solution. Samples of representative specimens were taken for electron microscopy. These were cut into very fine pieces, fixed in 4% glutaraldehyde for 24 hours at 4° C and then transferred to 0.05% cacodylate buffer before further processing.

Specimens for light microscopy were processed in a standard manner, embedded in wax and representative 5 µm sections cut. Sequential sections were stained with haematoxylin and eosin (H&E) and Ziehl Neelson (ZN) stains. The coded sections were read by the same observer (ICM) and demarcated as 'negative' or 'positive' depending upon the presence of granulomas on H&E staining. The presence or absence of ulceration and its site was also noted in the bowel specimens. If granulomatous infiltration was present then its extent was measured using a projection microscope and planimeter.

In the case of the lymph node, the areas of infiltration and the total area of the section, were traced on to a white sheet from the projected image (×36 magnification). The total area of the section and the infiltrated areas were measured using a fixed

arm planimeter (1 rev=100 cm²; constant=18 728) and the area of infiltration expressed as a percentage of the total area. In the case of the bowel sections, a similar procedure was followed, but a single representative field containing the inoculation site, together with all the granulomatous infiltration, was chosen and measured in an identical manner. Statistical assessment of data was by the Student's *t* test. All Ziehl Neelson sections were scanned completely and the presence or absence of acid fast bacilli noted.

Results

CLINICAL OBSERVATION

Animals in all the experiments were observed on a daily basis. They all gained weight normally and none showed any signs of clinical disease.

SKIN TESTS

These findings are given in Table 1. Inoculation with live BCG in either the terminal ileum or ascending colon, induced a significant reaction to purified protein derivative at two weeks $p<0.002$ and $p<0.001$ respectively. The response was greater after inoculation in the ascending colon although this difference did not reach a significant level. Animals inoculated in the terminal ileum also showed a further enhancement of response at five weeks but on analysis this increase was not found to be significant.

Table 1 Skin reaction to 25 µg of intradermal (purified protein derivative) after 24 hours expressed as increase in skinfold thickness mm-1 (SD)

	Increase in skinfold thickness (mm)
BCG in ascending colon at 2/52*	1.43 (0.8) $p<0.002$
BCG in terminal ileum at 2/52*	1.2 (0.8) $p<0.001$
BCG in terminal ileum at 5/52†	2.3 (0.5) $p<0.005$
Irradiated BCG ascending colon 5/52*	1.97 (0.4) $p<0.001$
Irradiated BCG terminal ileum 5/52†	3.0 (0.2) $p<0.001$
Irradiated BCG terminal ileum 2/52†	2.3 (0.6) $p<0.001$
Irradiated <i>M Leprae</i> ascending colon 5/52*	0.73 (0.5) $p<0.05$
Irradiated <i>M leprae</i> terminal ileum 5/52*	1.06 (0.6) $p<0.01$
N-Saline terminal ileum 2/52†	0.025 (0.04)
N-Saline terminal ileum 5/52†	0.075 (0.13)

p=Comparison with normal values at same time integer using Student's *t* test; *Standard deviations of groups of 12 animals; †Standard deviations of groups of six animals.

Table 2 Mean visceral lymph node weights (mg) (SD)

	Ileocolic lymph node	Caecal lymph node
BCG at 2/52 in ascending colon*	137.3 (66)	142.6 (46)
	p<0.01	NS
BCG at 2/52 in terminal ileum*	82.3 (30)	207 (59)
	NS	p<0.001
BCG at 5/52 in terminal ileum†	117.8 (21)	226.5 (93)
	NS	NS
Irradiated BCG 5/52 ascending colon*	223.7 (128)	318.1 (144)
	NS	NS
Irradiated BCG 5/52 terminal ileum†	265.8 (212)	648.8 (233)
	NS	p<0.01
Irradiated BCG 2/52 terminal ileum†	101.1 (23)	288.6 (102)
	p<0.05	p<0.01
Irradiated <i>M leprae</i> 5/52 ascending colon*	159.1 (51)	275 (87)
	p<0.02	p<0.02
Irradiated <i>M leprae</i> 5/52 terminal ileum*	130.6 (25)	271.6 (73)
	p<0.05	p<0.02
N-Saline 2/52 terminal ileum†	91 (4)	114 (20)
N-Saline 5/52 terminal ileum†	125 (31)	208 (64)

p=Comparison with normal values at same time integer using Student's *t* test; NS=Not significant; *Standard deviations of groups of 12 animals; †Standard deviations of groups of six animals.

Inoculation with irradiated BCG yielded a significant response at both sites and time integers $p<0.001$, which was significantly greater at five weeks $p<0.001$ and more pronounced in those inoculated in the terminal ileum than the ascending colon $p<0.002$. Animals inoculated with irradiated *M leprae* in the terminal ileum or ascending colon showed a much smaller, but still significant, response at 5/52 $p<0.01$ and $p<0.05$ respectively.

LYMPH NODE WEIGHTS

These findings are given in Table 2. In live BCG inoculated animals the draining lymph node weights were significantly raised at two weeks after inoculation in the ascending colon and terminal ileum, $p<0.005$ and $p<0.001$ respectively. This effect had, however, disappeared by five weeks. Animals inoculated with irradiated BCG at either site showed a non significant increase in the draining lymph node weight at both two and five weeks. Animals inoculated with irradiated *M leprae* showed a significant increase in both ileocolic and caecal node weights at 5/52.

GRANULOMATOUS INFILTRATION AT THE

INOCULATION SITE, MEASURED BY PLANIMETRY
Granulomatous infiltration expressed as a percentage of the representative histological section area is given in Table 3. At two weeks animals inoculated with live BCG showed a significant ($p<0.005$) difference in the degree of infiltration at the two inoculation sites,

the ascending colon being much more heavily infiltrated than the terminal ileum. Infiltration in the terminal ileum was past its peak at five weeks though not significantly less than at two weeks. A similar pattern was seen in the animals inoculated with irradiated BCG; infiltration continued to increase between two and five weeks in the terminal ileum though the difference was not statistically significant. There was no significant difference between the infiltration at the two inoculation sites in the case of irradiated *M leprae*. No granulomas were seen in the liver or spleen sections from any animal.

ULCERATION

Ulceration of the bowel mucosa overlaying the inoculated Peyer's patch, but nowhere else, was seen microscopically in some animals in all the groups, apart from the control animals. It occurred in 50% of the animals inoculated with live BCG in the ascending colon and in 27% inoculated with *M leprae* at the same site. Otherwise ulceration was seen only occasionally.

LYMPH NODE INFILTRATION

Granulomatous infiltration expressed as a percentage of the representative histological section area is given in Table 3. Infiltration was present in all cases in both ileocolic and caecal lymph nodes, apart from the control group, and was always at its greatest in the node immediately draining the inoculation site. There was no significant difference between the infiltration of the immediately draining node for any given organism, inoculation site, or time integer.

Table 3 Bowel and lymph node infiltration as percentage of representative field and total histological section respectively (SD) measured by planimetry

	Bowel infiltration	Ileocolic lymph node infiltration	Caecal lymph node infiltration
BCG ascending colon 2/52*	40.5 (15)	36.1 (12)	28.2 (14)
BCG terminal ileum 2/52*	21.5 (14)	16 (15)	24.4 (13)
BCG terminal ileum 5/52†	6.23 (4)	13.2 (13)	13.7 (5)
Irradiated BCG ascending colon 5/52*	44.7 (19)	41.3 (28)	16.9 (13)
Irradiated BCG terminal ileum 5/52†	34.1 (31)	55.9 (42)	73.2 (24)
Irradiated BCG terminal ileum 2/52†	28.4 (14)	32.1 (28)	66.9 (20)
Irradiated <i>M leprae</i> ascending colon 5/52*	39.7 (17)	43.6 (26)	34.6 (23)
Irradiated <i>M leprae</i> terminal ileum 5/52*	36.9 (7)	33.5 (10)	62.5 (14)

*Standard deviation of groups of 12 animals; †Standard deviation of groups of six animals.

Fig. 2 Electronmicrograph of epithelioid cell from granuloma induced 21/52 after inoculation of terminal ileum with live BCG (Pasteur). $\times 7500$.

ZIEHL NEELSON STAINING

Examination of Ziehl Neelson stained sections showed that with a single exception all bowel sections from animals inoculated with live BCG in the ascending colon showed the presence of acid fast bacilli at this site. Conversely, only one animal inoculated with live BCG in the terminal ileum showed the presence of acid fast bacilli. The only other positive findings were the bowel sections of one of 12 animals inocula-

ted with irradiated *M leprae* in the ascending colon at five weeks and in one of the immediately draining lymph nodes of these animals.

ELECTRON MICROSCOPY

Ultrastructurally the granulomatous tissue from live BCG inoculated animals revealed the presence of epithelioid cells, characterised by the presence of prominent large nuclei and endoplasmic reticulum in

Fig. 3 Electronmicrograph of macrophage infiltration 5152 after inoculation of terminal ileum with irradiated *M leprae*. $\times 3000$.

a cytoplasm otherwise largely devoid of organelles. Few acid fast bacilli were seen in either bowel or lymph node tissues (Fig. 2). Animals inoculated with irradiated BCG show the presence of a lesser number of epithelioid cells with similar characteristics. Fibroblasts with collagen deposition, however, were prominent. Tissue from animals inoculated with irradiated *M leprae* shows an infiltration of macrophages containing phagocytosed bacilli and cytoplasmic vacuoles (Fig. 3).

Discussion

This work shows that it is possible to produce a simple animal model of granulomatous infiltration in the bowel wall. Early attempts to achieve this were based upon the lymphoedema resulting from the direct inoculation of sclerosing reagents into the lymphatics draining the bowel¹ or on the piezo electric effect of finely divided silica materials administered by enteral feeding.² A reproducible model of an haemorrhagic

colitis has been induced in a number of animal species by the feeding of a carrageenan extract of seaweed.³ The resulting ulceration of the caecum, colon, and rectum bears some resemblance to ulcerative colitis in man, but it is noteworthy that patients receiving a regular intake of degraded carrageenan in the course of treatment for peptic ulcer disease show no increased incidence of inflammatory bowel disease.¹⁶

Homogenates of human Crohn's disease tissue inoculated into the rabbit ileum, after a prolonged period, in some cases, show changes in the bowel wall not dissimilar to Crohn's disease.⁴ This effect was destroyed when the homogenates were exposed to cobalt irradiation (2.5 MR) or passed through a 25 µm filter, thereby suggesting the presence of a viable and transmissible aetiological agent. Efforts to reproduce this work, however, have met with mixed success.^{5,6}

No information has hitherto been available regarding the results of direct inoculation of measured amounts of known mycobacteria into the bowel wall. It is relevant that among the many different factors that have been considered in relation to the aetiology of inflammatory bowel disease the role of a possible mycobacterial infection has received much attention. Thus Burnham *et al*⁸ isolated what were thought to be cell wall defective forms of mycobacteria from biopsy material of cases of inflammatory bowel disease and White *et al*¹⁷ using an ELISA technique were able to show increased levels of antimycobacterial antibodies in the sera of patients from whom they had isolated cell wall deficient organisms. Cheodini and Van Kruningen⁷ isolated from two patients with Crohn's disease a previously unrecognised acid fast mycobactin dependent mycobacterium, which was considered to be most closely related to mycobacterium paratuberculosis. A seven day old goat fed orally 50 mg of this organism developed granulomatous disease of its small intestine with the presence of small non-caseating tuberculoid granulomas at five months. Similarly Graham *et al*⁹ isolated cell wall defective forms of mycobacteria from 12 patients with Crohn's disease and three with ulcerative colitis; none was isolated from control samples. It is thus possible that mycobacteria not readily able to be cultivated with present techniques or that remnants from previous mycobacterial infection may play an important role in the aetiology of inflammatory bowel disease.

In our present study it is of interest that using live BCG the degree of granulomatous infiltration induced in the bowel varies so markedly depending upon whether the small or large bowel is involved. This was an unexpected but consistent finding. The mechanism underlying this is obscure but it is in keeping with the findings of Morson *et al*¹⁸ who noted

that the number of granulomas seen in a series of patients with Crohn's disease affecting either the terminal ileum or colon was always greater in the latter.

The differences seen in Ziehl Neelson staining are also relevant in this context. Acid fast bacilli are not a feature of Ziehl Neelson staining of Crohn's disease tissue and this has been used as an argument against their involvement in the aetiology of the condition. Our findings indicate that different areas of the bowel may handle mycobacteria differently and that even after the relatively short time intervals used in this study AFB may not be readily identified in mycobacteria induced granulomatous infiltrates. In this context it is of interest to note that Butcher *et al*¹⁹ described their negative findings after the use of cloned antimycobacterial DNA probes on human Crohn's disease tissues. In the absence of detectable DNA sequences using these sensitive and specific techniques they concluded that if mycobacteria are involved in the aetiology of Crohn's disease they either do not persist in diseased tissue or are present at a level of less than one mycobacterium per ten cells. Our findings show a further similarity to Crohn's disease in that although the inoculum was placed subserosally the majority of the resultant granulomatous infiltration occurred in the Peyer's patch and spread outwards into the surrounding submucosa. This is noted to be characteristic of Crohn's disease.²⁰ Moreover when ulceration occurred it was only in the mucosa overlaying the Peyer's patch and in this regard it is of interest that Hadfield in 1939²¹ noted a preponderance of ulceration in this region and postulated that it was due to pressure from the enlarging patch. In our study ulceration was most common in animals inoculated in the large bowel but not always in those with the most extensive bowel infiltration. Fissuring and true 'cobblestoning' of the bowel mucosa were not seen. This may be related to the relatively short time span of our study. We note that skin reactions to purified protein derivative were present after inoculation with live and irradiated BCG with a lesser response after the inoculation of irradiated *M leprae*. This is in contrast with previous work¹¹ which showed a greater skin test response with the same dose of purified protein derivative, to live BCG and irradiated *M leprae* at similar doses and time intervals when inoculated into the guinea pig ear. It is of interest that patients with Crohn's disease have in some studies,²²⁻²⁴ but not in others,²⁵ been shown to possess a defect of delayed cutaneous sensitivity on the basis of skin testing with purified protein derivative. Our findings from this study suggest that the presence of mycobacteria within the bowel wall may not give rise to the expected degree of delayed cutaneous sensitivity. The consistent and

reproducible animal experimental model we have described has enabled us to study the effects of a variety of immunomodulating agents on these mycobacteria induced granulomatous lesions. In the light of the findings of Cheodini and Van Kruningen,⁷ Burnham *et al*,⁸ White *et al*,¹⁷ and Graham *et al*⁹ it might be considered relevant to apply this technique to the study of the histological and cutaneous responses accruing from the inoculation of the principal strains of *Mycobacterium paratuberculosis* as well as investigating the effects of filtrates prepared from the granulomatous bowel and lymph node tissues of the present study.

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