Effect of the immune modulating agents cyclophosphamide, methotrexate, hydrocortisone, and cyclosporin A on an animal model of granulomatous bowel disease

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Abstract

This study was undertaken to determine the effect of cyclophosphamide, methotrexate, hydrocortisone, and cyclosporin A on a model of granulomatous infiltration in the terminal ileum and draining lymph nodes of the guinea pig. Treatment groups of six animals were used and compared to untreated groups of 12. Epithelioid cell granulomas and primary macrophage granulomas were induced by the inoculation of BCG (Pasteur) and irradiated *Mycobacterium leprae* respectively into the terminal ileum of the guinea pig. The response to purified protein derivative of tuberculin was reduced in both groups of animals receiving any of these agents. Cyclophosphamide and methotrexate treated animals inoculated with BCG or *M leprae* showed a significant reduction of granulomatous infiltration at the inoculation site ($p < 0.05$ and $p < 0.001$ respectively). BCG inoculated animals treated with either hydrocortisone or cyclosporin A showed no reduction in granulomatous infiltration at either the inoculation site or the draining lymph nodes. By contrast *M leprae* inoculated animals receiving either of these agents showed a significant reduction of granulomatous infiltration at both the inoculation site ($p < 0.001$) and in the primary draining lymph node ($p < 0.001$). Ziehl Neelsen staining showed an increased proportion of animals with detectable acid fast bacilli (AFB) at the inoculation site in the groups receiving hydrocortisone (50%) and methotrexate (67%) compared to untreated controls (8%). No AFB were observed in any of the animals inoculated with *M leprae*. In conclusion, this model may be helpful in elucidating the mechanism of T lymphocyte response in Crohn's disease and the variable clinical response seen with the use of immunosuppressive agents in this condition.

We recently reported on an animal model of granulomatous bowel disease¹ created by the direct inoculation of mycobacteria into the bowel wall of the guinea pig. Using this system we have been able to produce infiltration of the inoculation site and draining lymph nodes with either an epithelioid cell granuloma using BCG or a predominantly macrophage granuloma using irradiated *Mycobacterium leprae*. This model has enabled us to undertake a study of the effects on these differing types of granuloma, induced in

the gastrointestinal tract, of a variety of immune modulating agents acting at varying stages in the immune response.

Cyclophosphamide and methotrexate were chosen as their effects on the immune system in the guinea pig have already been extensively investigated.^{2,3} Hydrocortisone is of interest because prednisolone was the first drug shown by controlled trials⁴ to be effective in the treatment of Crohn's disease, and, with other glucocorticoids in systemic and topical forms, has remained a mainstay of treatment. Cyclosporin A was studied because of its unique mechanism of action⁵ and its putative role^{6,7} as a second line agent in the treatment of refractory or relapsed Crohn's disease.

Materials and methods

ANIMALS

Outbred Hartley strain female guinea pigs weighing 300 to 430 g were used. They were fed on an RGP pelleted diet supplemented with cabbage.

MYCOBACTERIA

Live BCG was of the Pasteur strain obtained by courtesy of the Institut Pasteur, Paris. Cobalt irradiated armadillo derived *M leprae* was obtained through the 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 Misra⁸ for viable organisms. *M leprae* were counted by the method of Hart and Rees,⁹ which gives a count of the total number of intact organisms. Live *M leprae* were not used because of legal restrictions owing to its pathogenicity in humans.

LAPAROTOMY

Laparotomy was performed using a procedure that we developed and fully described in a previous publication.¹ Briefly, animals were anaesthetised and using an aseptic technique laparotomy was performed through a midline incision. This approach gives ready access to the terminal ileum. The abdomen was closed using Vicryl sutures (Ethicon UK), the skin closure being reinforced with Histoacryl tissue adhesive (B Braun Melsungen AG, West Germany).

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Accepted for publication
4 September 1989

INOCULATION

All inoculations were of 50 µl and standard doses of organisms were used – namely, 2×10⁷ BCG and 2×10⁶ irradiated *M leprae*. Inoculation was performed using a Hamilton microlitre syringe and a 32 G needle into the serosa of the antemesenteric wall of the bowel at the level of the terminal ileal Peyer’s patch.

The doses used were based on those used in earlier work¹ in which they produced a satisfactory granulomatous infiltration in the terminal ileum and its draining lymph nodes and induced sensitivity to 25 µg of purified protein derivative of tuberculin (PPD) on skin testing. This dose produces no response in the unsensitised guinea pig.¹⁰

CONTROLS

Control animals were inoculated identically but received no drug treatment.

DRUGS

Cyclophosphamide (Endoxana, Boehringer Ingelheim Hospital Division) was given at a rate of 10 mg/kg body weight daily by intraperitoneal injection. Methotrexate sodium solution for injection (Lederle Laboratories) was given at a dosage of 5 mg/kg every second day by intraperitoneal injection. Hydrocortisone sodium succinate (Efcortelan, Glaxo Laboratories) was given as a solution in water subcutaneously at a dose of 0.6 mg/g body weight daily. Cyclosporin A was supplied courtesy of Dr J Borel (Sandoz, Basel, Switzerland) and given at a dose of 50 mg/kg body weight daily as an intramuscular injection in 0.05 ml absolute alcohol to avoid the use of oil based solvents. A further group of animals received 0.05 ml absolute alcohol daily as a control.

All animals apart from those in the hydrocortisone group underwent laparotomy on day 0 and started drug treatment on day 2. Animals receiving hydrocortisone were treated from day 0 with this drug before laparotomy and inoculation with mycobacteria on day 14. All drugs were continued to the time of harvesting.

The doses of cyclophosphamide and methotrexate were based on those known to produce a loss of the delayed hypersensitivity reaction to picryl chloride and 2 phenyl-4-ethoxymethylene-5-oxazolone (oxazolone) in guinea pigs previously sensitised to them.³

The doses of hydrocortisone and cyclosporin

A were based on those previously shown¹¹ to produce a significant depression of the response to 25 µg PPD in guinea pigs inoculated with an identical dose of BCG in the ear over a similar time span. The doses of all the drugs are in the human therapeutic range.

SKIN TESTS

Twenty four hours before harvesting (see below), the right flank of each animal was shaved and 25 µg of dialysed PPD was injected intradermally. The delayed hypersensitivity reaction was read at 24 hours using Schnelltester Kröplin callipers A 02T to measure the increase in skinfold thickness. The results are expressed as specific increases in skinfold thickness, which represent the reading (10⁻¹ mm) at the skin test site, minus the average thickness of the normal skin on both sides of the skin test site.

HARVESTING

Animals were killed two weeks after inoculation in the case of BCG and at five weeks in the case of *M leprae*. After terminal anaesthesia the abdomen was reopened through the original incision. After macroscopic inspection the inoculated area of bowel was excised, trimmed, and placed in 10% formol saline as were representative sections of the liver and spleen.

The ileocolic and caecal lymph nodes draining primarily the ascending colon and the terminal ileum respectively were excised, weighed, and prepared for fixation in Carnoy’s solution.

Specimens were processed in a standard manner, embedded in wax, and representative 5 µm sections cut. In previous studies using this technique¹ no significant increase in the area of granulomatous infiltration recorded was achieved by examining multiple sections. However, in this study where the area of granulomatous infiltration was found to be greatly reduced, further sections were cut through the block to ensure that the relevant area had not been missed. Sequential sections were stained with haematoxylin and eosin and Ziehl Neelsen stains. The coded sections were read by the same observer (ICM) and demarcated as negative or positive depending on the presence of granulomas on H&E staining. If granulomatous infiltration was noted then its extent was measured using a projection microscope and planimeter.

In the case of lymph node sections, the areas of granulomatous infiltration and the total area of section were traced on to a white sheet using the projected image (×36 magnification). The total area of section and the infiltrated area were measured using a fixed arm planimeter (1 rev= 100 cm², Constant 18728) and the area of granulomatous infiltration was expressed as a percentage of the total area. With bowel sections, a single representative field containing the inoculation site and all the granulomatous infiltration was chosen and this was measured in an identical manner. Student’s *t* test was used for statistical assessment of data. All the Ziehl Neelsen stained sections were scanned completely and the presence or absence of acid fast bacilli noted.

TABLE I Reaction to 25 µg of intradermal purified protein derivative of tuberculin after 24 hours, expressed as mean (SD) change in skinfold thickness (mm)

	2×10 ⁷ BCG in terminal ileum	2×10 ⁶ irradiated <i>M leprae</i> in terminal ileum
Cyclophosphamide 10 mg/kg intraperitoneally daily*	0.11 (0.12) p<0.01	0.033 (0.05) p<0.005
Methotrexate 5 mg/kg intraperitoneally on alternate days*	0.083 (0.12) p<0.01	0.25 (0.3) p<0.01
Hydrocortisone 0.6 mg/g subcutaneously daily*	0.18 (0.14) p<0.01	0.18 (0.18) p<0.01
Cyclosporin A 50 mg/kg in 0.05 ml absolute alcohol intramuscularly daily*	0.33 (0.5) p<0.05	0.14 (0.18) p<0.01
Absolute alcohol 0.05 ml intramuscularly daily*	0.525 (0.54) not significant	
Untreated†	1.2 (0.8)	1.06 (0.6)

*Mean (SD) of six animals; †Mean (SD) of 12 animals.
p=probability by comparison with untreated controls by Student’s *t* test.

TABLE II Mean (SD) visceral lymph node weights (mg)

	2 × 10 ⁷ BCG inoculated into the terminal ileum		2 × 10 ⁶ irradiated <i>M leprae</i> inoculated into the terminal ileum	
	Ileocolic node	Caecal node	Ileocolic node	Caecal node
Cyclophosphamide 10 mg/kg intraperitoneally daily*	86.5 (22) NS	154 (32) NS	53.3 (11) p<0.01	79.3 (24) p<0.001
Methotrexate 5 mg intraperitoneally on alternate days*	69.2 (7) NS	132.8 (0) NS	110.2 (28) NS	208.3 (50) NS
Hydrocortisone 0.6 mg/g subcutaneously daily*	78.3 (17) NS	172 (52) NS	63 (6.7) p<0.05	137.1 (25) p<0.01
Cyclosporin A in 0.05 ml absolute alcohol 50 mg/kg intramuscularly daily*	73.8 (6) NS	157.5 (34) NS	83.6 (15) NS	140 (26) p<0.01
Absolute alcohol 0.05 ml intramuscularly daily*	82.8 (11.7) NS	181.8 (32.7) NS		
Untreated†	82.3 (21)	207 (59)	130.6 (25)	271.6 (73)

*Mean (SD) of six animals; †Mean (SD) of 12 animals.

NS=not significant in comparison with untreated controls by Student's *t* test.p=probability in comparison with untreated controls by Student's *t* test.

Results

CLINICAL OBSERVATION

Animals in all the experimental groups were observed daily. None showed signs of clinical disease.

SKIN TESTS

Table I gives the skin test findings. Inoculation with BCG and subsequent treatment with any of the immune modulating agents caused an inhibition of the response to PPD (p<0.01 except in the case of cyclosporin A when p<0.05). Similarly, after inoculation with irradiated *M leprae* subsequent treatment with any of the agents produced an inhibition of the response to PPD (p<0.01 except in the case of treatment with cyclophosphamide when p<0.005). This inhibition was not observed in animals treated with absolute alcohol alone.

LYMPH NODE WEIGHT

Table II gives the mean lymph node weights. There was no reduction in either ileocolic or caecal lymph node weight in any of the BCG inoculated animals compared to the untreated controls. By contrast, animals inoculated with irradiated *M leprae* showed a significant reduction in caecal lymph node weight when treated with cyclophosphamide (p<0.001), hydrocortisone (p<0.01), or cyclosporin A (p<0.01). A significant reduction was also noted in the weight of the ileocolic node in the animals receiving

cyclophosphamide (p<0.01) or hydrocortisone (p<0.05).

GRANULOMATOUS INFILTRATION AT THE INOCULATION SITE AND PRIMARY AND SECONDARILY DRAINING LYMPH NODES (Table III)

Animals inoculated with BCG showed a significant reduction in granulomatous infiltration at the inoculation site after administration of either cyclophosphamide (p<0.05) or methotrexate (p<0.05). The latter group also showed a significant reduction of granulomatous infiltration of the caecal lymph node (p<0.05). No other groups inoculated with BCG showed any reduction of granulomatous infiltration.

By contrast, animals inoculated with irradiated *M leprae* showed a significant reduction of granulomatous infiltration both at the inoculation site (p<0.001) and in the caecal lymph node (p<0.001) with all the drug treatments. A reduction in the ileocolic lymph node weight was seen after treatment with cyclophosphamide (p<0.001) and cyclosporin A (p<0.01). No granulomas were observed in any of the liver or spleen sections.

ZIEHL NEELSEN STAINING

Animals inoculated with BCG showed an increased proportion with Ziehl Neelsen stained positive tissues at the inoculation site and in the draining lymph nodes when either hydrocortisone or methotrexate was administered.

TABLE III Terminal ileal and lymph node granulomatous infiltration as percentage of a representative field and total histological section respectively measured by planimetry (mean (SD))

	2 × 10 ⁷ BCG inoculated into the terminal ileum			2 × 10 ⁶ irradiated <i>M leprae</i> inoculated into the terminal ileum		
	Terminal ileum	Ileocolic node	Caecal node	Terminal ileum	Ileocolic node	Caecal node
Cyclophosphamide 10 mg/kg intraperitoneally daily*	5.1 (5)	4.5 (5.4)	20 (23)	0	0	0
	p<0.05	NS	NS	p<0.001	p<0.001	p<0.001
Methotrexate 5 mg intraperitoneally alternate days*	5.1 (12)	9.4 (15)	10.4 (9)	0	19.1 (22)	16.8 (22)
	p<0.05	NS	p<0.05	p<0.001	NS	p<0.001
Hydrocortisone 0.6 mg/g subcutaneously daily*	18 (16)	8.1 (7)	17.4 (19)	3.93 (7)	23.2 (22)	3.9 (7)
	NS	NS	NS	p<0.001	NS	p<0.001
Cyclosporin A 50 mg/kg in 0.05 ml absolute alcohol intramuscularly daily*	9.7 (15)	11.3 (11)	13 (7)	0	0	0
	NS	NS	NS	p<0.001	p<0.01	p<0.001
Absolute alcohol 0.05 ml intramuscularly daily*	12.2 (13)	12.1 (17)	14.3 (6)			
	NS	NS	NS			
Untreated†	21.7 (14)	16 (15)	24.4 (13)	36.9 (7)	33.5 (10)	62.5 (14)

*Mean (SD) of six animals; †Mean (SD) of 12 animals.

NS=not significant in comparison with untreated controls by Student's *t* test.p=probability by comparison with untreated controls by Student's *t* test.

TABLE IV Acid fast bacilli in tissues with Ziehl Neelsen staining (No positive/No sections)

	2×10 ⁸ BCG inoculated into terminal ileum			2×10 ⁸ irradiated <i>M leprae</i> inoculated into the terminal ileum		
	Terminal ileum	Ileocolic node	Caecal node	Terminal ileum	Ileocolic node	Caecal node
Cyclophosphamide 10 mg/kg intraperitoneally daily	1/6	0/6	0/6	0/6	0/6	0/6
Hydrocortisone 0.6 mg/g subcutaneously daily	3/6	1/6	1/6	0/6	0/6	0/6
Methotrexate 5 mg intraperitoneally alternate days	4/6	3/6	3/6	0/6	0/6	0/6
Cyclosporin A 50 mg/kg in 0.05 ml absolute alcohol intramuscularly daily	0/6	0/6	0/6	0/6	0/6	0/6
0.05 ml absolute alcohol intramuscularly daily	0/6	0/6	0/6			
Untreated	1/12	0/12	0/12	0/12	0/12	0/12

However, no increase was seen in the other groups whether BCG or *M leprae* was inoculated (Table IV).

Discussion

We used a previously delineated model¹ of epithelioid and phagocytic macrophage granulomas in the gastrointestinal tract of the guinea pig induced by the direct inoculation of BCG and *M leprae* respectively. These studies showed that after inoculation of live BCG into the ascending colon or terminal ileum a granulomatous infiltration was induced at the inoculation site and the draining lymph nodes. This granulomatous infiltrate was significantly greater in the large bowel compared to the terminal ileum for a given inoculum. No significant difference in the extent of granulomatous infiltration was seen between the two inoculation sites when *M leprae* was used.

Acid fast bacilli were present with Ziehl Neelsen staining of sections of the large bowel granulomatous infiltrate after BCG inoculation, but only rarely in sections from the terminal ileal lesions. There was a corresponding reduction in sensitivity to skin testing with PPD in animals inoculated in the small bowel. This is in contrast to the results of previous work¹² which showed a greater skin test response with the same dose of PPD to live BCG and irradiated *M leprae* at similar doses and time intervals when inoculated in the guinea pig ear.

The model was used to observe the effect of four immune modulating agents on granuloma formation in the gut. Cyclophosphamide and methotrexate reduced the areas of infiltration of both types of granuloma in the gut. Hydrocortisone and cyclosporin A reduced the area of infiltration of the phagocytic macrophage granuloma, but had no effect on the area of epithelioid granuloma infiltration. The response to PPD was reduced in all the treatment groups investigated.

Studies of the effect of cyclophosphamide and methotrexate on contact sensitivity in the guinea pig²³ showed that if cyclophosphamide was started within two days of sensitisation then this was inhibited. There was a drop in the proportion of large pyroninophilic cells or T immunoblasts in the local lymph node at a time when the T lymphocytes are known to go into their maximum proliferative phase.

Similar experiments using methotrexate again showed blockage of contact sensitivity but the action appeared to occur not directly on the large

pyroninophilic cells but on the T cells that developed from them. In our experiments an increased number of animals in the group treated with methotrexate after inoculation with BCG showed acid fast bacilli, but this was not observed in the *M leprae* inoculated group. This may represent a drop in host resistance which could be related to decreased T lymphocyte function. Our findings are thus largely in keeping with those from earlier studies on contact sensitivity. It is perhaps surprising that cyclophosphamide had no effect on lymph node infiltration in the BCG granuloma. It is possible that this represents a difference in sensitivity or in the control process of the T lymphocyte subsets involved in the response to BCG. Previous studies using monoclonal antibodies on a model of similar granulomas induced in the posterior auricular lymph node of the guinea pig by the inoculation of these mycobacteria into the ear¹³ showed the presence on the epithelioid cells of macrophage specific antigen but little evidence of major histocompatibility complex class II antigen. In contrast, the macrophages of the *M leprae*-induced granuloma expressed both macrophage specific and class II antigen. It thus appears that the epithelioid cells of a BCG induced granuloma share at least a close relationship with other cells of the mononuclear phagocyte series. A distinction between phagocytic macrophages and epithelioid cells in sarcoidosis and leprosy has also been observed in humans.¹⁴

Studies carried out using similar regimens of hydrocortisone and cyclosporin A on the BCG granuloma in the guinea pig ear model¹¹ showed that both reduced the skin test response to PPD. Neither agent, however, had any effect on the area of infiltration of the epithelioid cell granuloma. These findings agree with our observations. The macrophage granuloma induced by *M leprae* was not studied. Electron microscope studies showed reduction in the rough endoplasmic reticulum of the epithelioid cells and this was considered suggestive of an effect of hydrocortisone and cyclosporin A on the maturation of macrophages into epithelioid cells. This alteration in epithelioid cell morphology on electron microscopy may account for the increased number of animals seen in the hydrocortisone treated group with acid fast bacilli detectable with Ziehl Neelsen staining in both the bowel wall and the draining lymph nodes. By contrast in the experiments using the guinea pig ear model¹¹ the only acid fast bacilli observed were degraded organisms seen with the electron microscope in the cyclosporin A treated group.

It has been postulated that epithelioid cell maturation may be lymphokine mediated,¹⁵ although published reports are contradictory regarding the effects of glucocorticoids on lymphokine production and the ability of macrophages to respond to them.¹⁶⁻¹⁹ The reduced response to *M leprae* after treatment with hydrocortisone seen in our experiments may be related to a reduced level of prostaglandin E₂ and leucotrienes, which act as macrophage activators. They are believed to play an important part in modulating the immune response in ulcerative colitis^{20,21} and levels have been shown to correlate well with disease activity and fall towards control levels after treatment with prednisolone.²²

Cyclosporin A is believed to exert its effects by selectively influencing the clonal expansion and activation of individual T lymphocyte subsets by blocking the synthesis or release of interleukin 1 and interleukin 2 from monocytes and T helper cells respectively.²³ It also favours the expansion of antigen specific suppressor T cells²⁴ and thus acts at an early stage in the immune response leaving the established immunological memory intact.²⁵

Guinea pigs treated with cyclosporin A have been shown to have a reduced production of lymphokines *in vitro*²⁶ and this may explain the reduced response to PPD. The lack of effect on infiltration of an epithelioid cell granuloma in the bowel wall is also in keeping with the finding that prolonged treatment with cyclosporin A failed to prevent the formation of new granulomas in mice receiving subcutaneous implants of hepatic schistosomal granulomas.²⁷ Here some T lymphocyte function had returned by three weeks, suggesting that cyclosporin resistant clones had been stimulated. These results are in keeping with the results of the treatment of Crohn's disease with cyclosporin in humans first reported in 1984,⁶ where present results indicate a variable rate of response with relapse being common after treatment is stopped.^{7,28}

A marked reduction in macrophage activating factor has been noted in mice treated with higher doses of cyclosporin A (70 mg/kg/day).²⁹ It is possible that this effect on lymphokine production is responsible for the failure of formation of a macrophage granuloma in response to *M leprae* in our model.

It is of interest that, clinically, Crohn's disease shows a considerable variation in its response to treatment with a variety of immunosuppressive agents. The lack of response to hydrocortisone and cyclosporin A seen in the live BCG induced bowel granulomas, which has the closest histological resemblance to Crohn's disease in our models, may be analogous to this.

In conclusion, it may be that the variation in response achieved clinically in patients with Crohn's disease using either glucocorticoids or cyclosporin A represents the overall balance of effect on a series of T lymphocyte subsets with differing potentials and susceptibilities. The trial of other therapeutic agents used in the treatment of clinical Crohn's disease, together with further work using monoclonal antibodies against differing T cell subsets might be of value in elucidating these observations.

We wish to thank Miss M Jacques, Mr F Schindler, and Mr P Papasavva for their excellent technical assistance, and Mrs J Saxby for typing the manuscript. We also gratefully acknowledge the financial support of the Lilian May Coleman Fund of the Royal College of Surgeons of England.

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