Immunological studies in patients with Crohn's disease¹

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SUMMARY An investigation of immunological parameters was conducted in 38 patients with Crohn's disease. The immunological tests employed included skin tests with dinitrochlorobenzene and a battery of common skin test antigens, lymphocyte transformation with phytohaemagglutinin and pokeweed mitogen, serum immunoglobulins, and absolute lymphocyte counts. Crohn's disease patients were divided into two groups, those treated with immunosuppressive drugs and those not receiving immunosuppressive medications. The latter group was subdivided into patients with active and inactive disease. Immunosuppressed patients with Crohn's disease did not develop sensitivity to dinitrochlorobenzene and had mildly depressed skin test reactivity to common skin test procedures. Non-immunosuppressed patients with active Crohn's disease also reacted less frequently to common skin test antigens, but 16 of 17 such patients developed sensitivity to dinitrochlorobenzene. Lymphocyte transformation with phytohaemagglutinin and pokeweed mitogen was normal in all groups of patients with Crohn's disease. However, when suboptimal incubation periods were used with phytohaemagglutinin stimulation, there was a significant difference between Crohn's disease patients and controls. Serum immunoglobulin levels and absolute lymphocyte counts were normal in all Crohn's disease patients. We conclude that immunity in Crohn's disease is qualitatively normal.

Disagreement persists in the literature regarding the role of immunodepression in the aetiology and pathogenesis of Crohn's disease. The assessment of immunological function in Crohn's disease has been approached using a variety of methods including the response to skin test antigens (Phear, 1958; Binder *et al.*, 1966; Fletcher and Hinton, 1967; Bolton *et al.*, 1974) lymphocyte transformation with nonspecific mitogens (McHattie *et al.*, 1971; Parent *et al.*, 1971; Aas *et al.*, 1973; Asquith *et al.*, 1973; Guillou *et al.*, 1973; Sachar *et al.*, 1973; Bird and Britton, 1974) lymphocyte transformation in mixed lymphocyte cultures (Richens *et al.*, 1974), and enumeration of circulating B and T lymphocytes (Röpke, 1972; Bird and Britton, 1974). In several instances, evidence for immunological impairment has been found based on the results of these tests. However, an equal number of conflicting

results, using the same methods, has been obtained such that the influence of immunological factors in Crohn's disease remains unclear. Most previous studies have limited themselves to the use of a single method in assessing immunocompetence. We therefore undertook a comprehensive analysis of immunological function in our patients with Crohn's disease, correlating results obtained using several different methods. The results of these studies form the basis of this report.

Methods

PATIENTS

Thirty-eight patients with documented Crohn's disease were studied. Twenty-five patients were receiving azulfidine and/or antidiarrhoeal medication or no treatment at the time they were studied. Fourteen other Crohn's disease patients were receiving immunosuppressive agents consisting either of azathioprine, or prednisone, or both. One patient (B.S.) was studied twice, once while receiving no medication and once while receiving prednisone.

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The clinical characteristics of the non-immunosuppressed patients with Crohn's disease are listed in Table 1.

Disease activity was estimated using the criteria developed for the National Cooperative Crohn's Disease Study (Best *et al.*, in press). The variables assessed included pain, sense of well-being, fever, weight loss, number of stools per day, complications, haematocrit, and use of antidiarrhoeal medication. An activity index of 150 or greater was considered indicative of active disease.

Thirty-two patients with gastrointestinal diseases other than Crohn's disease and 52 normal volunteers served as controls. The gastrointestinal diseases represented included viral gastroenteritis, irritable colon, Whipple's disease, post-irradiation enteritis, post-gastrectomy for carcinoid tumour, bacterial overgrowth, short bowel syndrome, ulcerative colitis, coeliac sprue, giardiasis, lactase deficiency, peptic ulcer disease, and gastroenteritis which was not otherwise specified. Twenty-six patients with lymphoproliferative disorders (LPD) were included as a separate control group to document the ability of the lymphocyte transformation test to detect impairment of cellular immunity. In all instances, informed consent was obtained. These investigations

had the approval of the University of Vermont Committee on Human Experimentation.

SKIN TESTS

Dinitrochlorobenzene (DNCB) Sensitization to DNCB was performed according to the method described by Catalona *et al.* (1972). On day 0, 2000 μ g DNCB in 0.1 ml acetone was applied to a circular area of skin 1.2 cm in diameter on the volar aspect of the upper arm. Simultaneously, 50 μ g was similarly applied to the forearm skin. The sites were covered with gauze for 24 hours and examined daily for an allergic ('flare') reaction. After 14 days, if no flare reaction were observed at either primary sensitization site, the patient was challenged with 50 μ g DNCB applied to the skin of the opposite forearm. This site was observed for 48 hours for erythema, induration, oedema, and vesiculation. Reactions were graded according to the following scheme:

- 3+—flare reaction at both primary sensitization sites.
- 2+—flare reaction at 2000 μ g site only.
- 1+—allergic reaction at 50 μ g challenge site only.
- 0—no reaction at any site.

No.	Patient	Sex	Age (yr)	Treatment	Disease activity	Site of involvement
1	M.L.	F	22	None	Active	Terminal ileum and diffuse involvement of colon
2	T.B.	F	39	Tincture of opium Lomotil	Active	Descending colon, rectum
3	J.E.H.	M	27	Azulfidine	Active	Terminal ileum, descending colon
4	J.A.H.	M	32	Azulfidine Lomotil	Active	Ileocelectomy—1973
5	N.S.	M	48	Lomotil	Inactive	36 in terminal ileum and ascending colon resected—1973
6	E.W.	F	42	Azulfidine	Active	Terminal ileum; colon at hepatic flexure; rectum
7	S.T.	F	26	None	Active	Terminal ileum—15 in
8	M.O.	M	21	Lomotil	Active	Transverse colon and terminal ileum
9	S.A.	M	26	None	Inactive	Terminal ileum—16 in
10	K.H.	F	15	Azulfidine	Inactive	Descending colon
11	L.R.	F	66	Azulfidine	Inactive	Terminal ileum and right colon
12	D.C.	F	50	Tincture of opium	Inactive	Terminal ileum
13	D.L.	M	37	None	Inactive	20 in terminal ileum resected—1967
14	J.C.	M	64	None	Inactive	Terminal ileum
15	J.F.	F	28	Tincture of opium	Inactive	Terminal ileum
16	M.B.	F	46	Tincture of opium	Inactive	Portion of terminal ileum and ascending colon, resected—1967
17	A.D.	F	19	Azulfidine	Inactive	10 in terminal ileum
18	N.T.	F	66	Lomotil	Inactive	Terminal ileum and right colon resected—1973
19	M.F.	M	23	Azulfidine	Inactive	Terminal ileum—12 in
20	B.S.	F	33	None	Inactive	25 in terminal ileum resected—1969
21	H.P.	M	60	Azulfidine	Inactive	Ileal resections 1960, 1969
22	R.C.	M	26	None	Active	Colon
23	R.D.	M	24	None	Active	Terminal ileum and colon
24	H.B.	M	30	None	Active	Terminal ileum and cecum
25	L.D.	M	28	None	Inactive	Terminal ileum

Table 1 Summary of patient data—non-immunosuppressed patients with Crohn's disease

Common skin test antigens The following skin test antigens were injected intradermally into the forearm skin in 0.1 ml volumes: PPD (Parke-Davis, intermediate strength), mumps skin test antigen (Lilly), candida (Hollister-Stier, Dermatophyton O 1:100), and streptokinase-streptodornase (Lederle, Varidase, 5 U/ml). The diameter of induration present 48 hours after inoculation was measured and values greater than 5 mm were considered to be positive reactions.

LYMPHOCYTE TRANSFORMATION

Measurements of tritiated thymidine uptake by to be whole blood culture technique (Pauly and Sokal, 1972) were used for quantitative assessments of lymphocyte reactivity. Ten millilitres of venous blood were drawn into plastic syringes containing 25 U sodium heparin (Upjohn beef lung heparin preserved with benzyl alcohol). Specimens were kept at room temperature and used within 18 hours. Absolute lymphocyte counts were performed. Cell suspensions were prepared for culture by diluting one volume of whole blood with 19 volumes of synthetic tissue culture medium (RPMI 1640, Grand Island Biological Co.) containing 100 U/ml penicillin and 100 µg/ml streptomycin. This dilution of whole blood (1:20) was determined by experiment to give adequate stimulation values and ease in subsequent solubilization procedures. No serum supplements were added. Cultures, usually in five-fold replicates, contained 3 ml cell suspension in disposable 16 × 100 mm glass tubes with nonsealing plastic closures. Reconstituted phytohaemagglutinin (Burroughs Wellcome Co., MR 68), 0.4 ml/culture, or pokeweed mitogen (PWM, Grand Island Biological Co.), 0.32 mg in 0.4 ml/culture, were added to appropriate replicate cultures to test for T cell and combined T and B cell reactivity respectively. After 96 hours incubation at 37°C in a 5% CO₂ humidified atmosphere, ³H-thymidine (sp. act. 1.9 Ci/mMol, New England Nuclear), 1.0 µCi per tube was added to each culture and incubated for another 24 hours. Cells were harvested by resuspending the cell button, transferring the cell suspension to centrifuge tubes, and rinsing the culture tubes with cold 3% acetic acid. The rinse was added to the centrifuge tubes and the tubes were filled with cold 3% acetic acid. The tubes were centrifuged at 150 g for 10 minutes. The cell button was washed once in cold acid, recentrifuged, and the supernatant solution decanted. One drop of 30% hydrogen peroxide was added to each of the cell buttons after which the pellets were warmed to 85°C for 20 minutes in a drying oven. Six-tenths of a millilitre of NCS tissue solubilizer (Amersham/Searle Corporation) was added to each centrifuge tube and the contents agitated. Ten

millilitres of a scintillation fluid solution composed of PPO (2.5 diphenyloxazole, New England Nuclear Co.) 22.7 g, POPOP (1.4 bis-2-[5-phenyloxazolyl] benzene) 0.0379 g, and 3.79 l toluene, were added to each centrifuge tube. The contents were then transferred to glass counting vials and the specimens counted in a Packard liquid scintillation spectrophotometer. Results are recorded in counts per minute (cpm), representing the mean values of replicated cultures and are expressed as (1) cpm in unstimulated cultures, (2) cpm in cultures after stimulation, and (3) the stimulation ratio before/after.

MISCELLANEOUS

Serum levels of IgG, IgM, and IgA were measured in the clinical laboratory by the radial immunodiffusion method. Absolute lymphocyte counts were calculated from the total white blood cell count and per cent lymphocytes in the differential count. The presence of isohaemagglutinins was determined for patients with blood groups A, B, O.

Results

SKIN TESTS

Dinitrochlorobenzene sensitization DNCB sensitization was evaluated in 17 non-immunosuppressed patients with Crohn's disease, seven patients with Crohn's disease who were receiving immunosuppressive drugs, and 13 controls (three normal volunteers and 10 patients with gastrointestinal disorders other than Crohn's disease). The results are tabulated in Table 2. All controls developed a flare reaction at one or both of the primary sensitization sites. In the group of non-immunosuppressed patients with Crohn's disease, 16 of 17 individuals developed sensitivity to DNCB at one or both of the primary sensitization sites. One patient (D.C.) in this group failed to react at either the primary sensitization sites or the challenge site. This patient was a 50 year old woman with Crohn's disease, involving the terminal ileum, of seven years' duration. Her disease was inactive at the time of this study. She reacted weakly to SK-SD and was PPD negative. Her lymphocyte transformation tests with PHA and PWM were normal (stimulation ratios of 212 and 40 for PHA and PWM respectively). All Crohn's disease patients immunosuppressed with prednisone or agathioprine failed to develop sensitivity to DNCB. In general, these patients also failed to develop the skin irritation usually seen within 48 hours after the application of DNCB in high doses.

Common skin test antigens The results of skin

	Crohn's disease			Immunosuppression	Controls
	No immunosuppression				
	Active	Inactive	Total		
DNCB	7/7	9/10	16/17	0/7	13/13
PPD	0/8	4/12	4/20	1/11	2/14
SK-SD	3/8	10/12	13/20	4/11	10/14
Mumps	4/8	6/9	10/17	4/6	7/10
Candida	3/8	6/9	9/17	4/6	7/10
Combined†	6/8	12/12	18/20	7/11	13/14

Table 2 Summary of skin test data*

*Numerator: number of positive reactions. Denominator: total number in group.

†Individuals reacting to at least one skin test antigen.

tests with common skin test antigens are summarized in Tables 2 and 3. Patients with active Crohn's disease reacted less frequently to these antigens than patients with inactive disease or controls. This trend was particularly apparent when the reactions to candida, SK-SD, and mumps were compared in the various groups. This difference was not of sufficient magnitude to reach statistical significance, however. When the results are combined to show the number of individuals reacting to at least one of the skin test antigens employed, the non-immunosuppressed patients with active Crohn's disease reacted slightly less frequently and less vigorously than patients with inactive disease or controls. Immunosuppressed patients also reacted less vigorously to the battery of common skin test antigens. However, seven of 11 such patients reacted positively to at least one of the antigens employed. The fact that none of these patients developed sensitization to DNCB illustrates the relative sensitivity of the primary immune response to immunosuppression in comparison with the secondary response. Positive reactions to PPD were infrequent in each group, demonstrating that PPD reactivity is not sufficiently widespread in the general

population to merit its use as a screening test of immunodeficiency.

LYMPHOCYTE TRANSFORMATION

Lymphocyte transformation in the presence of phytohaemagglutinin (PHA) or pokeweed mitogen (PWM) was tested in 24 non-immunosuppressed patients with Crohn's disease and nine patients with Crohn's disease who were receiving immunosuppressive agents. The control group consisted of 52 normal volunteers, 30 hospitalized patients with a variety of gastrointestinal disorders other than Crohn's disease, and 26 patients with LPD.

Table 4 summarizes the results of these studies. Patients with Crohn's disease did not differ from hospitalized control patients when either stimulation ratios or absolute counts in stimulated cultures were compared. This was true both for PHA and PWM. Immunosuppressive agents in the therapeutic regimen appeared to exert little influence on these results. Stimulation ratios for Crohn's disease patients and hospitalized controls did not differ significantly from those of normal controls. Also, the results from patients with active Crohn's disease did not differ from those obtained in patients with

Antigen	Crohn's disease							
	No immunosuppression				Immunosuppression			
	Active		Inactive				Controls	
	10 mm	5-9 mm	10 mm	5-9 mm	10 mm	5-9 mm	10 mm	5-9 mm
PPD	0/8	0/8	3/12	1/12	1/11	0/11	2/14	0/14
SK-SD	3/8	0/8	9/12	1/12	3/11	1/11	9/14	1/14
Mumps	3/8	1/8	3/9	3/9	3/4	0/4	6/10	1/10
Candida	1/8	2/8	4/9	2/9	1/4	1/4	2/10	5/10
Combined†	4/8	2/8‡	10/12	2/12‡	7/11	0/11‡	12/14	1/14‡

Table 3 Frequency and intensity of positive reactions to common skin test antigens*

*Numerator: positive reactions. Denominator: number of individuals in group.

†Individuals reacting to at least one skin test antigen.

‡Indicates number of individuals with weak reactions only in group.

Patient groups	Absolute lymphocyte count (ALC) (no./mm ³)	Unstimulated value (counts/min)	PHA stimulated value (counts/min)	PHA ratio†	PWM stimulated value (counts/min)	PWM ratio
Normal controls N = 52	3 200 ± 200	1 809 ± 197	124 884 ± 9 988	95 ± 11	32 190 ± 3 327	22 ± 3
Hospitalized GI controls N = 30	2 800 ± 200	577 ± 45	6 247 ± 9 352	108 ± 15	14 213 ± 1 987	25 ± 3
Lymphoproliferative disorders (LPD) N = 26	35 000 ± 13 000	3 414 ± 1 801	34 499 ± 6 891	43 ± 10‡	7 191 ± 2 433	9 ± 3§
LPD; ALC 5 500 N = 16	2 500 ± 300	1 154 ± 537	33 726 ± 9 985	54 ± 14	8 391 ± 3 912	10 ± 5
LPD; ALC 5 500 N = 10	83 000 ± 26 000	7 089 ± 4 497	35 736 ± 8 762	24 ± 12	5 271 ± 1 133	4 ± 2
Crohn's disease N = 33	2 800 ± 200	672 ± 66	65 540 ± 4 647	111 ± 8	15 473 ± 2 248	25 ± 3
Crohn's disease immunosuppression N = 9	2 900 ± 400	796 ± 121	66 863 ± 8 829	106 ± 9	18 625 ± 2 185	25 ± 3
Crohn's disease no immunosuppression N = 24	2 700 ± 200	626 ± 78	60 355 ± 5 352	113 ± 11	14 291 ± 2 971	26 ± 4

Table 4 Results of In vitro lymphocyte stimulation tests with phytohemagglutinin (PHA) and pokeweed mitogen (PWM)*

*Mean values ± one standard error of the mean.

†Ratio = stimulated value/unstimulated value.

‡P < .005 (Student's *t* test with Bonferroni correction made for three comparisons).

§P < .01.

inactive disease. Absolute counts in cultures stimulated by either mitogen were higher for the group of normal volunteers than for the groups of hospitalized patients. The explanation for this observation is not known.

Patients with LPD could be separated into two groups on the basis of absolute lymphocyte counts. The degree of stimulation by each mitogen was similar in both groups. However, unstimulated cultures from patients with absolute lymphocyte counts greater than 5 500 incorporated H³ thymidine to a greater degree than those with absolute lymphocyte counts less than 5 500. Stimulation ratios for the combined group of patients with LPD were significantly lower for both mitogens in comparison with the other groups. These results confirm the ability of this *in vitro* method to detect immunodepression in some clinical conditions.

Preliminary experiments, using a three day rather than a seven day incubation period for lymphocytes

stimulated with PHA, produced unexpected results in eight patients with untreated Crohn's disease compared with 11 normal controls (Table 5). Both absolute counts and stimulation ratios were significantly greater in stimulated cultures from the normal individuals than in the patients with Crohn's disease. This suggests that lymphocytes from Crohn's disease patients might be less responsive to mitogens initially, although they respond in a quantitatively normal fashion when conditions are optimal.

MISCELLANEOUS

Isohaemagglutinins were detected in the serums of all patients with blood groups A, B, and O. Serum immunoglobulins were measured in 27 patients with Crohn's disease, including 18 non-immunosuppressed patients and nine patients receiving steroids. The data obtained are given in Table 6. Serum IgA levels were modestly, but not significantly, elevated in patients with Crohn's disease who were not receiving

Patient group	Absolute lymphocyte count (ALC) (cells/mm ³)	Unstimulated value (counts/min)	Stimulated value (counts/min)	Ratio†
Normal controls N = 11	3 600 ± 400	3 978 ± 900	196 737 ± 38 067	56 ± 6
Crohn's disease N = 8	2 500 ± 400	3 133 ± 478	58 684 ± 15 640	24 ± 9‡

Table 5 Results of in vitro lymphocyte stimulation tests with normal individuals and patients with Crohn's disease: three day cultures*

*Mean values ± one standard error of the mean.

†Ratio—stimulated value/unstimulated value.

‡P < 0.005 (Student's *t* test).

	Crohn's disease		Ill controls (10)*	
	No immunosuppression (19)		Immunosuppression (9)	
	Active (8)	Inactive (11)		
Absolute lymphocyte count (no./mm ³)	2 275	1 524	2 342	1 271
IgG (mg%)	1 338	1 124	1 036	1 232
IgM (mg%)	126	92	99	115
IgA (mg%)	346	312	271	256

Table 6 Serum immunoglobulins and absolute lymphocyte counts in Crohn's disease patients and controls

*One patient with Waldenstrom's macroglobulinemia excluded.

steroids. The levels of IgG and IgM fell within the normal range for our laboratory. Elevation of serum IgA in Crohn's disease has been reported previously (Bolton *et al.*, 1974; Jones *et al.*, in press).

Absolute lymphocyte counts were calculated for the patients with Crohn's disease and controls. Patients with Crohn's disease tended to have higher lymphocyte counts than controls, excluding the patients with malignant LPD. Non immunosuppressed patients with inactive Crohn's disease showed no elevation of lymphocyte counts. These data are included in Table 6.

Discussion

The results reported here suggest that the aspects of the immune response which we measured in patients with Crohn's disease are qualitatively normal. A variety of immunological tests were employed, none of which differentiated between non-immunosuppressed patients with Crohn's disease and controls.

Attempts to sensitize patients with DNCB were successful in 94% (16 of 17) of individuals with Crohn's disease who were not receiving immunosuppressive therapy. In contrast, none of the immunosuppressed patients developed DNCB sensitization.

Verrier-Jones found that 58% of patients with Crohn's disease failed to develop sensitivity to DNCB and that this unresponsiveness was not correlated with disease activity (Jones *et al.*, 1969). However, Bolton *et al.* (1974) found that 80% of Crohn's disease patients developed sensitivity to DNCB similar to results in controls and in closer agreement with our studies.

The results of skin tests using the battery of common skin test antigens illustrate several points. Steroid-treated patients tended to be less responsive to these antigens than other patient groups. Nevertheless, many such patients continued to develop strong reactions despite steroid treatment. Patients with active Crohn's disease who were not receiving

immunosuppressive agents also reacted less intensely and less frequently to these antigens, but only two of eight such patients failed to react to at least one of the common skin test antigens. Both unreactive patients were relatively young (26 and 32 years respectively) and each developed an intense flare reaction at the high dose primary sensitization site with DNCB. Their failure to react to the recall antigens might reflect a lack of exposure to these antigens rather than immunological impairment. Binder also reported that patients with Crohn's disease reacted to skin test antigens as frequently as controls (Binder *et al.*, 1966).

The results of the lymphocyte transformation assays corroborate the skin test results. Only patients with lymphoproliferative disorders differed significantly from the other groups tested. Normal individuals tended to incorporate tritiated thymidine more readily in both mitogen stimulated and unstimulated lymphocyte cultures. Stimulation ratios for normal individuals, hospitalized controls, and Crohn's disease patients were similar. This was true whether their disease were judged to be active or not. Patients receiving steroids were similar to the other patient groups with regard to lymphocyte responsiveness *in vitro*. However, none of the steroid-treated patients developed a positive skin test reaction after sensitization with DNCB.

Experiments using the whole blood PHA-induced lymphocyte transformation technique revealed that short, suboptimal incubation periods produced stimulation ratios which discriminated between Crohn's disease patients and normal individuals. The optimal seven day incubation abolished this difference. This observation might explain some discrepant results which have appeared in the literature. Recently, investigators employing PNA-induced lymphocyte stimulation have emphasized the value of dose response curves studied over multiple time intervals in detecting immunological impairment. It should be noted that Röpke, in a study of patients with Crohn's disease, found no difference in lymphocyte kinetics after PHA

stimulation (Röpke, 1972).

The explanation for this observation is not known. Strickland *et al.*, in a recent study of T and B lymphocytes in peripheral blood of patients with Crohn's disease, found that T lymphocytes were modestly depressed (Strickland *et al.*, 1974). However, similar investigations by Bird and Britton failed to confirm this observation (Bird and Britton, 1974). Alternatively, hyporesponsiveness to mitogen stimulation in other conditions has occasionally been associated with serum inhibitors (Socia-Foca *et al.*, 1973), medications including both salicylates (Crout *et al.*, 1975) and steroids (Walker and Greaves, 1969), and malnutrition (Law *et al.*, 1973).

The explanation for the conflicting results reported in the literature regarding cell-mediated immunity in Crohn's disease is unclear and undoubtedly complex. Patients with Crohn's disease vary in their disease activity, duration of disease, age, nutritional status, and therapy. Each of these variables might influence the results of immunological tests. Moreover, our ability to translate the results of the crude immunological tests at our disposal into meaningful clinical correlations is extremely limited except in the most obvious of clinical situations such as the immune deficiency diseases and malignancies of the lymphoreticular system. Until more discriminating methods become available, we contend that immune depression in Crohn's disease has not been proven and that the role of depressed immunity in the pathogenesis of Crohn's disease is uncertain.

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