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Functional suppressor T cell activity in Crohn's disease and the effects of sulphasalazine

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SUMMARY

Suppressor T cell activity was measured in 18 patients with Crohn's disease and 20 controls, using two different functional assays. The effects of sulphasalazine and its metabolites on *in vitro* suppressor cell activity were also studied. The activity of a Con A-induced suppressor cell system in patients with Crohn's disease did not differ from that of controls, suggesting that the previously reported abnormalities are a secondary phenomenon. Furthermore, the activity of a non-induced suppressor T cell system was also normal in these patients. There was no evidence either *in vivo* or *in vitro* to suggest that sulphasalazine exerts its beneficial action by an effect on this aspect of immunoregulation.

INTRODUCTION

The findings of various abnormalities of the immune response in inflammatory bowel disease have led a number of investigators to suggest that immune mechanisms are involved in the pathogenesis of the disease, and several analogies have been drawn with diseases in which immune or autoimmune mechanisms are known to be involved (Sachar, Auslander & Walfish, 1980; Kraft, 1979; Whorwell & Wright, 1978). Following the discovery that lymphocytes could be subdivided into several subgroups, including suppressor and helper T cells, Hodgson, Wands & Isselbacher (1978) described reduced suppressor T cell activity in inflammatory bowel disease and suggested that this defect might allow for potentially damaging immune responses directed against a variety of candidate antigens to continue unchecked. Similarly there have been preliminary reports that intestinal lymphocytes from patients with Crohn's disease exhibit abnormal suppressor T cell activity compared to those from controls (Goodacre & Bienenstock, 1980; Fiocchi, Battioto & Farmer, 1979; Fiocchi, Youngman & Farmer, 1981). As the mode of action of sulphasalazine, a drug of known benefit in inflammatory bowel disease, is unknown, we postulated that it may have an action on immunoregulatory function. The aim of this study was to try to confirm the presence of reduced T cell suppressor cell activity in Crohn's disease and to investigate the possibility that sulphasalazine or its metabolites may exert beneficial effects by an action on this limb of the immunoregulatory system.

PATIENTS AND METHODS

Subjects. Eighteen patients with Crohn's disease were studied. All had had their diagnoses

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confirmed radiologically or histologically and the disease had been present for at least 3 months. Fourteen were in remission, four in relapse. Six were taking corticosteroids and eight sulphasalazine. Patients in remission were all out-patients with a Crohn's disease activity index (CDAI, Best *et al.*, 1979) of less than 150. Patients in relapse were all in-patients with a CDAI of more than 300. A total of 20 healthy volunteers from hospital and laboratory staff served as a control group. The two groups were of similar age range and sex distribution. All patients gave informed consent and the study conformed to the guidelines of the Committee on Human Experimentations at the University of Vermont.

Preparation of peripheral blood mononuclear cells (PBMC). PBMC were obtained by density centrifugation after layering fresh heparinized venous (10 units/ml) blood on Ficoll-Hypaque. Mononuclear cells were recovered and washed twice with phosphate-buffered saline (PBS). Cell suspensions were made in RPMI 1640 with 25 mM HEPES (GIBCO) and supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml) and 2 mM glutamine (Microbiological Associates) (RPMI-PSG). Trypan blue exclusion was used to assess viability and any preparation less than 95% viable was discarded.

Concanavalin A (Con A) suppression of T cell activity (Con-ASST). Con A suppression of T cell activity was based on the method of Shou, Schwartz & Good (1976), which measures the degree of inhibition of Con A-induced ^{3}H-thymidine incorporation in responder T lymphocytes by Con A-generated suppressor T cells. In order to generate suppressor T cells, peripheral blood mononuclear cells were adjusted to a concentration of 5×10^6 in RPMI-PSG and incubated with 20% human AB serum in 1 ml aliquots in round-bottomed tubes with 60 μg/ml Con A (Calbiochem) for 48 hr. Identical cultures were incubated without Con A for subsequent addition to responder lymphocytes so that the 'non-suppressed' Con A-induced ^{3}H-thymidine incorporation of the latter could be assessed. Following the 48 hr incubation, the aliquots were recombined, pelleted and the cells suspended in fresh RPMI-PSG plus 50 μg/ml mitomycin C (Sigma Chemicals Co.) and incubated for 30 min. The cells were washed three times in α-methyl-*d*-mannoside (Sigma) in Hank's buffered saline solution (HBSS) (Microbiological Associates) to remove cell-bound Con A and then resuspended in RPMI-PSG prior to reincubation with responder lymphocytes.

Because the degree of suppression of responder lymphocytes may be dependent in part of the responder cells themselves, two donors allogeneic to the suppressor cells were used as sources of Con A responder cells. In later experiments, cells isogeneic to the suppressor cells, i.e. autologous cells, were used also as Con A responders to avoid possible HLA restriction of cell interactions.

As noted, suppressor activity was measured by the inhibition of Con A (7 μg/ml) stimulated ^{3}H-thymidine incorporation in peripheral blood mononuclear responding cells by Con A-generated suppressor cells. Suppressor cells (10^5) and responder cells (10^5) were incubated in microtitre plates at 0·2 ml/well with 20% human AB serum for 3 days at 37°C in a humidified 5% CO_2 atmosphere. Cells were labelled with 1 μCi/well ^{3}H-thymidine (NEN sp. act. 6·7) 18 hr prior to harvesting on glass fibre filters in a Titertek Cell Harvester (Flow Lab). Dried filters were counted in minivials by liquid scintillation spectrometry in a Packard Tri-Carb.

The data are expressed as mean c.p.m. from triplicate cultures and the degree of suppression calculated according to the following formula:

$$\% \text{ suppression} = 1 + \frac{\text{c.p.m.}_{\text{s}} \cdot \text{Con A-c.p.m.}_{\text{s}} \cdot \text{O}}{\text{c.p.m.}_{\text{c}} \cdot \text{Con A-c.p.m.}_{\text{c}} \cdot \text{O}} \times 100$$

in which c.p.m.$_s$. Con A is mean c.p.m. of ^{3}H-thymidine incorporation in responder cell cultures containing Con A-generated suppressor cells (in addition to responding cells) and Con A, c.p.m.$_s$. O is ^{3}H-thymidine incorporation in cultures containing Con A-generated suppressor cells but no Con A, c.p.m.$_c$. Con A is ^{3}H-thymidine responder cell incorporation in responder cell cultures containing pre-incubated control cells (in addition to responding cells) and Con A, and c.p.m.$_c$. O is ^{3}H-thymidine incorporation in responder cell cultures containing pre-incubated control cells but no Con A.

Effects of sulphasalazine and its metabolites. Sulphasalazine (SS), sulphapyridine (SP) and 5-amino-salicylic acid (5-ASA), all supplied by Pharmacia, were used at a concentration of 100 μg/ml. Their effect on the generation of suppressor cells in the pre-incubation period of 48 hr was

assessed, both by themselves *in lieu* of the Con A and also with co-incubation with Con A. The cells were otherwise treated exactly as for the standard Con A assay. The concentrations of the drugs used approximate serum levels achieved in clinical practice. The effects of the drugs were measured only in the isogeneic system.

The non-induced suppressor T cell system (NSS). Non-induced suppression of T cell activity was based on the method of Hong *et al.* (personal communication), described in full elsewhere (Krawitt *et al.*, 1981). This measures the ability of irradiated T lymphocytes obtained from the test subject to inhibit a one-way mixed leucocyte culture (MLC). Cultures were in microtitre plates. Responder and stimulator cells used in the MLCs were the same in all assays, and were allogeneic to the test suppressor cells. Each well contained 10^5 responder cells, 10^5 irradiated stimulator cells, 10^5 irradiated test cells ('suppressor T cells'), and 25% human AB serum; control cultures were the same except that they contained 10^5 irradiated responder cells *in lieu* of irradiated suppressor cells. The cultures were incubated for 6 days at 37°C in a humidified 5% CO_2 atmosphere before labelling with 1 μCi/well ^{3}H-thymidine 18 hr prior to harvesting on glass fibre filters in a Titertek Cell Harvester (Flow Lab.). Dried filters were counted in minivials by liquid scintillation spectrometry in a Packard Tri-Carb.

Suppression was calculated from the difference between mean c.p.m. obtained in MLCs containing test suppressor cells and in control MLCs which contained only irradiated responder cells by the following formula:

$$\% \text{ suppression} = 1 - \frac{\text{c.p.m.}_s}{\text{c.p.m.}_c} \times 100$$

in which c.p.m.$_s$ is the mean c.p.m. of the MLC in the presence of test irradiated suppressor cells and c.p.m.$_c$ is the mean c.p.m. of the MLC in the presence of irradiated responder cells.

Statistical analysis. The Student's *t*-test was used for statistical analysis. The unpaired *t*-test was employed for comparing groups and the paired *t*-test used for comparing the *in vitro* effect of the drugs. Probability values of less than 5% were considered significant.

RESULTS

The results of both suppressor T cell assays in patients and controls are shown in Table 1. There was no difference between Con A-induced suppressor cell activity using either of the two allogeneic responders or in the isogeneic system. Similarly, there was no difference in the non-induced suppressor cell activity. There was no clear cut relationship to therapy or disease activity. None of the Crohn's disease patients were outside 2 s.d. of the mean of the normals. The four patients in relapse (all hospitalized and on treatment with corticosteroids) also showed normal Con A-induced suppressor activity (68% ± 14, 54% ± 28 and 72% ± 32 for responder I and II and for isogeneic cells respectively). The respective values for the eight patients on sulphasalazine were 64% ± 26, 58% ± 24 and 68% ± 14.

The effects of the addition of sulphasalazine and its metabolites SP and 5-ASA on the generation

Table 1. Results of suppressor cell assays in controls and patients with Crohn's disease

	Control	Crohn's
Con A-induced suppressor activity		
Allogeneic responder 1	67·2 ± 19·4 (20)	68·9 ± 25·3 (16)
Allogeneic responder 2	62·8 ± 17·9 (20)	58·5 ± 23·7 (14)
Isogeneic responder	54·7 ± 21·3 (20)	60·2 ± 25·2 (14)
Non-induced suppressor activity	−102 ± 109	−88 ± 117 (18)

Results expressed as % (mean ± s.d.). Figure in parenthesis indicates number studied.

Table 2. Effects of adding sulphasalazine (SS) and its metabolites, sulphapyridine (SP) and 5-amino-salicylic acid (5-ASA) to the preincubation period *in lieu* of Con A

	Medium alone	SS	SP	5-ASA
Patient ($n=9$)	31,125±1,515	27,976±2,156	28,561±1,642	23,487±2,005
Controls ($n=8$)	56,243±2,584	52,234±3,307	50,864±2,686	53,957±3,415

Results expressed as c.p.m. (mean±s.d.).

of suppressor cells are shown in Table 2, which indicates the effects of the drugs alone, and in Table 3, which indicates the effects of adding the drug with Con A during generation of suppressor cells. The addition of the drugs to the system during the 48 hr generation period made no significant difference either in the patients with Crohn's disease or in the controls, whether added alone or with the Con A.

Table 3. Effects of adding sulphasalazine (SS) and its metabolites sulphapyridine (SP) and 5-amino-salicylic acid (5-ASA) to Con A preincubation in the Con A isogeneic suppressor cell system.

	Con A alone	Con A +SS	Con A +SP	Con A +5-ASA
Crohn's ($n=9$)	50·8 ±14·2	44·4 ±28·2	48·5 ±28·7	59·8 ±20·7
Control ($n=8$)	38·9 ±21·1	41·5 ±11·5	37·4 ±28·7	41·2 ±19·5

Results expressed as % (mean±s.d.).

DISCUSSION

Our results showing normal Con A-induced suppressor cell activity in Crohn's disease are in direct contrast to those of Hodgson (Hodgson *et al.*, 1978), who found reduced Con A suppressor cell activity in four out of five patients with active Crohn's disease. As far as we are aware, the only other report of abnormal Con A-induced suppression in Crohn's is that of Knapp *et al.* (1981), who found reduced levels in eight patients in relapse but normal activity in six patients in remission.

Although we used different concentrations of Con A, particularly during the first incubation, this should not account for the differences, and the methods are otherwise similar. We did not encounter the problems of leuco-agglutination reported by Hodgson *et al.* (1978) at this higher dose, which is the concentration used in the original report of the method (Shou *et al.*, 1976). It is noteworthy that the range of suppression generated in our normal controls was similar to that reported by Hodgson *et al.* (1978), and that we have shown reduced suppressor cell activity in chronic active hepatitis using this same system (Krawitt *et al.*, 1981). The assay is in part HLA-dependent and results do differ between different responders (Krawitt *et al.*, 1981). To avoid this problem, unlike the previous workers who used only one allogeneic responder, we have used two allogeneic responders, as well as isogeneic responder cells. We have also found no significant difference using a non-induced assay of suppressor cell activity. Technical differences might account for some of these discrepancies, but it seems most likely that the previously reported abnormalities of Con A-induced suppression indicate a transient, or secondary, abnormality which is related to some variable, such as disease severity or drug therapy. This conclusion is also reached by Pfreundschuh *et al.* (1981) in a study utilizing membrane marker techniques.

We consider that the failure of this study to show any reduced suppressor T cell activity in any of our responder systems is strong evidence against there being a primary defect in Crohn's disease. Further evidence to support this emerges from a study of the conflicting reports of both increased T cell suppression of immunoglobulin synthesis (Elson *et al.*, 1981) and reduced T cell suppression as measured by a non-induced assay of suppressor T cell activity dependent upon the observation that suppressor T cells are inactive after a short culture period (Victorino & Hodgson, 1981). Similarly, there are reports of increased (Fiocchi *et al.*, 1981) and reduced (Goodacre & Bienenstock, 1980) Con A-induced suppressor cell activity by intestinal mononuclear cells. More recently, techniques employing surface markers and monoclonal antibody techniques have been introduced to differentiate between helper and suppressor T lymphocytes. To our knowledge, two groups have employed these techniques in Crohn's disease and report increased T_M (helper), but normal T_G (suppressor) cells (Victorino & Hodgson, 1980a,b; Pfreundschuh *et al.*, 1981). This would also seem to support our finding of normal suppressor T cell activities although the relationship of these assays to the functional assays is not straightforward (Victorino & Hodgson, 1980a,b). In other diseases, notably scleroderma, directly conflicting results were reported using similar techniques of membrane markers by two different groups (Gupta *et al.*, 1979; Inoshita *et al.*, 1981), and further confirmation of these findings in Crohn's disease is required.

Despite claims to the contrary, drug therapy in Crohn's disease remains relatively empirical and corticosteroids and sulphasalazine are the two major drugs employed. Corticosteroids have anti-inflammatory properties and are known to be immunodepressant. Hydrocortisone inhibits the generation of suppressor cells when added to the Con A system, although this may be a non-specific effect (Knapp & Posch, 1980). The mode of action of sulphasalazine is unknown, but it has been suggested that its beneficial effect is due to anti-inflammatory (Das *et al.*, 1973), antibiotic (West *et al.*, 1974), or anti-prostaglandin (Sharon *et al.*, 1978) activity. In this study, no effect of sulphasalazine on the aspects of immune regulation studies were detected, either *in vitro* or *in vivo*. The concentrations of the drugs used in this study were similar to those which are known to inhibit lymphocyte proliferation *in vitro*, and it is therefore perhaps surprising that the addition of the drug to the Con A system did not inhibit the induction of suppressor cells. However, other studies on the effect of inhibitory drugs have shown that those drugs which inhibit mitosis or protein synthesis do not inhibit the response, while only those that inhibit microtubule or microfilament function have such an inhibitory effect (Shand, Orme & Ivanyi, 1980). The observation that 5-ASA, which had been shown in several studies to be the active moiety of the drug (Azad Khan, Piris & Truelove, 1977; Van Hees, Bakker & Van Tongeren, 1980; Klotz *et al.*, 1980), does not behave differently from SP, which is thought to be an inert carrier, is further support for the lack of any effect of this drug on the T cell suppressor cell systems studied in this report. It has previously been shown by others that SS does not affect the total number of circulating T or B cells present, although helper/suppressor cell ratios were not studied in this report (Thayer, Charland & Field, 1979).

In conclusion, we have not been able to confirm that there is abnormal Con A-induced suppressor T cell activity in Crohn's disease. The growing number of conflicting reports of both increased and decreased suppressor T cell activity, together with our findings, suggests that any defect is likely to be a secondary phenomenon. In this study there is no evidence either *in vivo* or *in vitro* that sulphasalazine or its metabolites exert their beneficial effects by an action on this limb of the immunoregulatory system.

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