Immune status in Crohn's disease¹

2. Originally unimpaired primary cell mediated immunity *in vitro*

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SUMMARY One-way mixed lymphocyte cultures (MLC) were performed with peripheral blood lymphocytes of 21 patients with Crohn's disease (CD) not receiving salizylazosulphapyridine, steroids, or azathioprine, seven patients with inflammatory bowel disease other than CD and ulcerative colitis, and 46 age- and sex-matched normal control subjects. The group of CD patients consisted of 11 patients with newly diagnosed, short-standing and so far untreated CD (group CD 1) and 10 patients previously treated with drugs and with mostly long-standing CD (group CD 2). Results showed that the MLC responsiveness was similar in all Crohn's disease groups, normal subjects and diseased controls. While there was no correlation between MLC responsiveness and either disease activity or disease duration when compared singly, those CD 2 patients who had highly active and/or very long-standing disease did exhibit a depressed MLC responsiveness as compared with that of normal subjects ($P < 0.01$), CD 1 patients who had both inactive and short-standing disease ($P < 0.05$), and diseased controls ($0.1 \geq P > 0.05$). The stimulatory capacity did not differ significantly between the CD groups and normal subjects or diseased controls; the latter, however, stimulated poorly compared with normal subjects ($P < 0.05$). In accordance, an inverse relationship between the magnitude of the stimulatory capacity and the disease activity was found in the CD patients as a whole. These data suggest that there is no depression of the *in vitro* primary cell mediated immune response as a predisposing factor for CD or as an early event associated with the pathogenesis of CD.

Divergent data regarding the numerical distribution as well as the functional status of the circulating thymus derived lymphocytes (T cells) have been reported in Crohn's disease (CD). However, there seems to be wide agreement that the primary cell mediated immunity (CMI) is defective in CD, based on the finding of an impaired skin sensitisation to dinitrochlorobenzene (DNCB; Jones *et al.*, 1969; Meuwissen *et al.*, 1975; Meyers *et al.*, 1976). Also, the *in vitro* responsiveness of lymphocytes to allogeneic lymphocytes in the mixed lymphocyte culture (MLC), which is considered to represent an *in vitro* model of the primary CMI, has been found to be impaired in CD patients (Richens *et al.*, 1974).

We have recently shown (Auer *et al.*, 1976; Auer *et al.*, 1978) that in a group of CD patients, which consisted exclusively of newly diagnosed patients

whose disease was of short-standing and who had not been treated with drugs (group CD 1), there was no numerical defect of the carriers of CMI, but that there was a significant reduction of the absolute number of T lymphocytes in a group of CD patients with long-standing disease and/or a history of drug treatment (group CD 2). This study revealed that a reduction of the number of T cells occurs in the disease. However, more or less normal numbers of T cells do not necessarily indicate normal T cell function. In the hope of answering the question whether the alleged impairment of the primary CMI response in CD patients is (1) present early in CD and thus might possibly be primary, predisposing to the disease, or (2) is secondary to influences as they occur during the disease, we studied the MLC reactivity of peripheral blood lymphocytes (PBL) in two CD groups defined according to the above mentioned criteria.

Methods

PATIENTS AND CONTROLS

In all instances informed consent was obtained

¹Supported by Deutsche Forschungsgemeinschaft Au 35/2, Au 35/4, and Au 35/5.

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before testing. At the time of being tested none of the patients was on such drugs as salicylazosulphapyridine (SASP), steroids, or azathioprine (AZA).

Patients with Crohn's disease

To exclude any influence of bowel resection *per se* on the MLC reactivity, only patients without any history of bowel resection were included in this report. None of the patients had had any surgery within the last month. The diagnosis of Crohn's disease was confirmed histologically in 11 of the 21 CD patients. Disease activity was estimated according to Best *et al.* (1975). A Crohn's disease activity index (CDAI) ≤ 110 was considered to indicate quiescent-mild, an index of 111 to 200 a moderately active, and one of over 200 a highly active disease.

The 21 Crohn's disease patients as a whole were evaluated in group CD, which, in fact, was composed of two subgroups—namely, group CD 1 and CD 2. Group CD 1 consisted of a selected consecutive series of 11 CD patients, who had been newly diagnosed at time of the study and who had never been treated by SASP, steroids, or azathioprine. Crohn's disease was usually of short standing. The duration of illness showed a mean of 29.6 ± 9.2 months. The disease was restricted to the ileum in four cases, to the small bowel as well as the colon in five, and to the colon in two cases. Seven patients had mild disease, one a moderately active and three a highly active disease. The mean age was 33.9 ± 4.7 years, with a range from 21 to 67 years. This rather high mean age was mainly caused by two patients, whose disease was newly diagnosed at 58 and 67 years, respectively. Without these two patients the age mean would have been 27.5 ± 2.4 years. Group CD 2 comprised 10 patients, the disease of most of whom had been diagnosed long before the onset of this study (mean of the duration of illness: 53.1 ± 10.1 months). Nine patients had been treated with SASP. In six of them therapy included steroids and one was also treated by azathioprine. All drugs were withdrawn three to 10 weeks before testing. Four patients had disease limited to the ileum, five patients had ileocolitis, and one colonic disease only. Three patients had mild disease, two patients a moderately active and five a highly active disease. The mean age was 26.5 ± 2.0 years.

Controls

Both healthy subjects (N groups) and a group of patients with inflammatory bowel diseases other than Crohn's disease or ulcerative colitis (CU; group D) were studied as controls. Two age- and sex-matched normal control subjects were tested simultaneously with each patient. They had been bled at the same time of the day as the patients in order to allow for

the circadian rhythm of T cell reactivity (Kaplan *et al.*, 1976). These normal controls made up the groups NCD 1, NCD 2, NCD, and ND. Group D comprised seven patients who suffered from diverticulitis coli (two), salmonellosis gastroenteritis (two), bacterial overgrowth (two) and gastroenteritis, which was not otherwise specified (one). The mean age was 23.1 ± 4.0 years.

LEUCOCYTE SEPARATION

Leucocytes were separated as previously described (Auer and Kress, 1977). The cells for mixed lymphocyte cultures were divided into two parts, one of which was given 4500 r irradiation by a ^{60}Co -source and was used as a source of stimulating cells.

LYMPHOCYTE CULTURES

One-way mixed lymphocyte cultures (MLC) were performed by the microculture technique in U-bottomed wells as previously described (Auer and Kress, 1977), with minor modifications. In brief, triplicate MLCs contained 1×10^5 responder (res) cells and 1×10^5 stimulator (stim) cells per well in a total volume of 0.2 ml. Control cultures contained 1×10^5 responder cells and 1×10^5 x-irradiated autologous cells per well in 0.2 ml. The cells were suspended in RPMI 1640 (GIBCO, Grand Island, N.Y.) plus HEPES (25 mM), supplemented with penicillin (50 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), L-glutamine (2 mM), and 10% heat inactivated (30 minutes at 56°C) AB pool (from healthy non-transfused males). Incubation was performed for six days in a humidified 5% CO_2 – 95% air atmosphere after preliminary experiments had revealed an optimal incubation period of 144 hours under the conditions of our laboratory. One microcurie of tritiated thymidine (^3H -TdR; spec. act. 2 Ci/mol, New England Nuclear, Boston, Mass.) was added for the last eight hours of culture. The cells were harvested with a semiautomatic harvesting machine (MASH II, Microbiological Associates, Bethesda, Maryland) and the radioactivity incorporated into DNA was evaluated as described previously (Auer and Kress, 1977).

Mixed lymphocyte cultures were performed using two normal control cultures and one or two CD and/or D patients, each set of cells being used both as stimulators (stim) and responders (res) with all cell types available. The mean of the results of two normal controls tested simultaneously was used as a reference for comparison with the corresponding CD or D patients. With regard to the patients, the mean of the results obtained in the same kind of combination with two unrelated normal subjects was used for calculations.

The results of the MLC experiments were ex-

pressed and calculated in the form of uncorrected cpm, corrected cpm, and in relative response. Corrected cpm were calculated using the formula: $\text{cpm AresBstim} - \text{cpm AresAstim}$, in which AresBstim represents cpm in the stimulated culture and AresAstim cpm in the autologous control culture. When the data were calculated in uncorrected cpm, results virtually identical with corrected cpm were obtained, the latter of which are, therefore, exclusively presented. Evaluation of results in relative response and relative stimulatory capacity, respectively, has been shown to be most suitable for comparison of results because it largely eliminates the effect of day-to-day variation in the level of [^3H]-TdR incorporation (Stastny, 1976). The formula for calculation of relative reactivity was as follows:

$$\text{Relative response (\%)} = \frac{\text{mean corr. cpm of stimulated patient cultures}}{\text{mean corr. cpm of stimulated normal control cultures}} \times 100$$

$$\text{Relative stimulatory capacity (\%)} = \frac{\text{mean corr. cpm of stimulating patient cultures}}{\text{mean corr. cpm of stimulating normal control cultures}} \times 100$$

The relative response or stimulatory capacity, therefore, was expressed as a percentage of the normal response or stimulatory capacity, both being obtained as the mean from the two normal control cultures.

BLOOD RESPONSE CAPACITY

The *in vitro* response of mixed lymphocyte cultures stands in gross relation to the proportion of MLC responsive T cells present in a given number of lymphocytes. To reach a closer relation to the *in vivo* conditions, where the absolute number of T cells might have, in general, a considerable bearing on the magnitude of the primary CMI, a response capacity per mm^3 blood (blood response capacity, BRC) was calculated as follows:

$$\text{BRC} = \frac{\% \text{ rel. response} \times 10^2}{\% \text{ of T cells} \times 10^5} \times \text{abs. number of T cells/mm}^3 \text{ blood (res-cells/well)}$$

in which the fraction corresponds to the relative response of a single responder cell in the culture.

T LYMPHOCYTES BINDING SHEEP RED BLOOD CELLS (E-ROSETTES)

E-rosettes, which are thought to represent T cells,

were determined as previously described (Auer *et al.*, 1978).

STATISTICAL ANALYSIS

The results are given as mean $\pm$ SEM, unless otherwise stated. Wilcoxon's matched pairs signed ranks test was used to compare the Crohn's disease patients with normal subjects, in that the data of single CD patients were compared with the mean of the data of the corresponding simultaneously tested sex- and age-matched normal control subjects. We thus paid attention to the known age and sex dependency of CMI parameters and to the inter-assay variations inherent in the MLC technique. For testing the significance of difference in the residual calculations Wilcoxon's two sample ranking test (U-test) was used. Correlations were studied by Spearman's rank correlation test.

Results

CONTROL CULTURES

As can be seen from Fig. 1, the [^3H]-TdR uptake in control cultures of group CD 1 consisting of a mixture of equal numbers of autologous responder and stimulator cells was slightly but significantly decreased compared with the corresponding normal subjects (NCD 1), but was comparable with the diseased controls (D), who, in turn, also showed slightly lower cpm than normal. There were no further significant differences in the control cultures between any of the groups (Fig. 1). The decrease in CD 1 had no influence on the outcome of the results in the MLCs.

When responder and stimulator cells were cultured separately, no significant differences in the spontaneous [^3H]-TdR uptake between any of the patients groups and the corresponding normal subjects of diseased controls were found.

RESPONSIVENESS AND STIMULATORY CAPACITY IN MLC

Random one-way cultures were performed in checker-board fashion in several experiments with a total of 21 CD patients, seven D patients, and 46 normal subjects. Thus, four combinations were usually obtained. Two of them consisted of normal responders stimulated either by other normal subjects (Nres vs. Nstim, referred to as responsiveness and stimulation capacity of normal subjects, respectively) or by patients (Nres vs. CDstim or Nres vs. Dstim, referred to as stimulation capacity of patients), and the other two were patient responders stimulated either by normal subjects (CDres vs. Nstim or Dres vs. Nstim, referred to as responsive-

Fig. 1 $[^3\text{H}]\text{-TdR}$ uptake in control cultures of the various groups of Crohn's disease patients and controls. ● single CD or D patient as indicated. ○ mean of results obtained from the two simultaneously cultured controls corresponding to the CD or D patients as indicated.

ness of patients) or, if there were two or more patients in the experiment, by other patients of the same disease group (CDres vs. CDstim).

The MLC responsiveness of the patient lymphocytes (CDres vs. Nstim; Dres vs. Nstim) in the various groups was comparable with that of the corresponding normal subjects (Nres vs. Nstim), both when expressed in cpm (Fig. 2a) and in relative response (Fig. 3a). The CD patient groups were also comparable with group D (Figs. 2a and 3a), except for the relative responsiveness in CD2 which was somewhat lower than in D and also than in CD 1 (Figs. 2a and 3a).

Unlike group D, which showed a stimulatory capacity (Nres vs. Dstim) significantly below normal (Nres vs. Nstim), the stimulatory capacity (Nres vs. CDstim) in the three CD groups did not differ significantly either from normal, from group D (Figs. 2b and 3b), or from each other.

When Crohn's disease patients were stimulated by other CD patients cpm as well as relative 'reactivity' were not significantly different from normal (Fig. 4a and b). However, in group CD 2, both considerably lower cpm (Fig. 4a) and a lower relative 'reactivity' were observed than in group CD 1 (Fig. 4b). One pair of CD 2 patients (patients A and B) showed a very low MLC 'reactivity' (AresBstim 32%; BresAstim 19%). This poor reactivity was mainly caused by patient B, who also exhibited both a very low stimulatory capacity and responsiveness when cultured with normal subjects.

INFLUENCE OF CD SERA ON MLC

When CD sera (diluted 1:2, 1:10 and 1:40) were added to MLCs of normal subjects, in only one of 13 CD sera tested a significant suppression (> 50% suppression) of the MLC occurred.

BLOOD RESPONSE CAPACITY (BRC)

The MLC responsiveness stands in gross relation to the proportion of MLC responsive T cells present in a given number of lymphocytes. Thus, the significant decrease of the absolute numbers of T cells/mm³ blood in group CD 2 as compared with normals (CD 2 (938 ± 100) vs. NCD 2 (1776 ± 136); $P < 0.01$) or group D (CD 2 vs. D (1907 ± 276); $P < 0.005$), which contrasts with close to normal values in group CD 1 (CD 1 (1464 ± 228) vs. NCD 1 (1611 ± 152)), is not reflected in these MLC results. Therefore, we tried to take that into account in calculating a 'blood response capacity' per mm³ blood (BRC). Thus, a possible hint might be obtained from the *in vitro* primary cell mediated immunity response on the *in vivo* conditions.

Separate evaluation of the two CD subgroups revealed (Fig. 5) that the depression of the BRC in group CD as a whole was caused by group CD 2, which was highly significantly depressed as compared with normal subjects as well as with group D, whereas group CD 1 gave results similar to both group NCD 1 and D.

RELATION TO CLINICAL PARAMETERS

No significant correlations emerged between the responsiveness in the MLC and clinical parameters as disease activity (CDAI) or disease duration, when correlated singly. However, when we compared the MLC responsiveness of those CD 2 patients who had a CDAI > 200 and/or a disease duration ≥ 49 months with that of those CD 1 patients who had both a CDAI ≤ 110 and a disease duration ≤ 12 months a markedly lower response was found in the former group ($P < 0.05$ for response in cpm; $0.1 \gg P > 0.05$ for relative response), the MLC response of which was also lower than in group D ($0.1 \gg P >$

Fig. 2 (A) MLC responsiveness (*Nres* vs. *Nstim*; *CDres* vs. *Nstim*; *Dres* vs. *Nstim*) and (B) MLC stimulation capacity (*Nres* vs. *Nstim*; *Nres* vs. *CDstim*; *Nres* vs. *Dstim*) of the various groups of Crohn's disease patients and controls, expressed in cpm. In both figures: ● mean of results obtained in the same kind of combination from a single CD or D patient, who were separately cultured with two normal subjects. ○ mean of the results obtained in the same combination from the two normal subjects corresponding to the CD or D patients as indicated.

Fig. 3 (A) MLC responsiveness (*Nres* vs. *Nstim*; *CDres* vs. *Nstim*; *Dres* vs. *Nstim*) and (B) MLC stimulation capacity (*Nres* vs. *Nstim*; *Nres* vs. *CDstim*; *Nres* vs. *Dstim*) of the various groups of patients, expressed in percent of normal subjects. In both figures: each circle represents the mean of results obtained in the same kind of combination from a single CD or D patient who was separately cultured with two normal subjects.

0.05 for cpm and relative response). The relative MLC response of these CD 2 patients was also significantly lower than that of the entire group of

normal subjects ($p < 0.01$). This CD 2 subgroup contained five of the six CD patients, who had previously been treated with steroids and the one

Fig. 4 MLC results of Crohn's disease patients stimulated by other CD patients (CDres vs. CDstim) and of normal controls (Nres vs. Nstim). (a) Results expressed in cpm. ● results obtained from a single CD patient. ○ mean of the results obtained in the same kind of combination from the two normal subjects corresponding to the CD patients as indicated. (b) Results expressed in percent of normal. Each circle represents the results obtained from a single CD patient.

patient who had received AZA.

In accordance with this trend, two of the three CD 1 patients with a CDAI over 200 and the CD 1 patient with the longest disease duration were found among the four CD 1 patients with a BRC below 1 SD of normal subjects (Fig. 5).

In group CD 1 and in group CD as a whole, the relative stimulatory capacity was found to be significantly lower in highly active patients (CDAI > 200) than in those with quiescent disease (CDAI ≤ 110; Fig. 6).

RELATION TO PROPORTION OF T-LYMPHOCYTES

No significant correlations were found between responsiveness and the proportion of T cells in any of the patient groups.

Discussion

As a first finding in the present work we encountered a subnormally low [^3H]-TdR uptake in the unstimulated mixed lymphocyte cultures of CD 1 patients. Since, however, the spontaneous thymidine incor-

poration of both CD-subgroups was comparable with that of group D, the decrease from normal in CD 1 was obviously a reflection of the disease state and cannot be interpreted as an indication for a specific state of 'anergy' early in Crohn's disease. This decrease in CD 1 is not comparable with that reported previously in PBL of Crohn's disease patients (Meuwissen *et al.*, 1975), since the control cultures for the mixed lymphocyte cultures contained mixtures of autologous responder and stimulator cells, whereas in Meuwissen's work only responder cells were tested. Those showed a normal [^3H]-TdR uptake in all groups in the present work.

The most important finding of this study on the *in vitro* parameter of the primary CMI responsiveness was that the MLC responsiveness of PBL in CD patients early in the disease and before any therapy has been started (group CD 1) is comparable with that of normal subjects as well as of patients with inflammatory bowel disease other than Crohn's disease or ulcerative colitis. These data contrast the report of a significantly decreased primary CMI response *in vitro* which was considered as reflection of a relative state of anergy in Crohn's disease

Fig. 5 Blood response capacity per mm^3 blood in Crohn's disease patients and diseased controls. Each point represents one individual. The mean value $\pm$ SD of the normal controls are indicated by the dotted area. For further details see text.

(Richens *et al.*, 1974). Richens used the stimulation index (SI) for evaluation of the mixed lymphocyte culture data. The stimulation index, however, places undue emphasis on control values, the standard error of which on replicate control cultures has repeatedly been found to be more than twice as high than that of the stimulated cultures (Thurman *et al.*, 1973; Herva, 1977). However, calculation of our data by means of the stimulation index gave similar results as before.

Among the factors that might have a bearing on this disparity in the results are the following: (a) the use of pooled normal AB serum instead of autologous plasma as done in Richens' study; (b) the exclusive use of patients off any drug treatment; and (c) the collection and evaluation of Crohn's disease patients separately according to the definitions of groups CD 1 and CD 2.

As far as (a) is concerned inhibitory serum factors on MLC reactions, as shown in systemic lupus erythematoses (Wernet *et al.*, 1974), have to be

Fig. 6 Relative stimulation capacity (Nres vs. CDstim) in the MLC in group CD in relation to disease activity (CDAI). Each point represents a single CD patient. ● CD 1 patients. ▲ CD 2 patients.

considered, as lymphocytotoxic autoantibodies have been described also in Crohn's disease (Korsmeyer *et al.*, 1974). However, we failed to find suppressive factors for the mixed lymphocyte culture in Crohn's disease sera in this study.

Where factors (b) and (c) are concerned, the only slight trend towards lower MLC responses in group CD 2 than in CD 1 and D, turned out to have, indeed, some relevance. A significant difference in the MLC responsiveness was found when the subgroup of those CD 2 patients with longest standing and/or highly active disease was compared with those CD 1 patients who had both very short duration and inactive disease, or with all normal subjects. This also indicates that in Crohn's disease there is a multifactorial influence on the *in vitro* primary CMI response, in form of disease activity, duration of disease, as well as a possibly long-lasting impairment of the primary CMI by immunosuppressive therapy. It is unclear, at present, whether this influence might act at the cellular level—for example, by a deficiency of folic acid which has been shown to be limiting in the MLC (Bain, 1975) or by the decrease of the proportion of MLC reactive T cells, due to sequestration and loss in the gut (Douglas *et al.*, 1976). The fact that no statistically significant correlation was found between the MLC

responsiveness and the proportion of T cells does not exclude the latter possibility, as disease processes may affect the functionally heterogeneous T cell subsets within the total T cell population to a different extent. So, most of Richens' patients with Crohn's disease not only fulfilled the criteria of our group CD 2, but were even on immunosuppressive therapy in contrast with all of our patients. These differences in the populations studied and/or technical differences might have lead to the disparity in the results that we have discussed.

The *in vitro* findings presented here might have a bearing on the repeatedly, though not unanimously, reported defect of the primary CMI *in vivo* (Jones *et al.*, 1969; Meuwissen *et al.*, 1975; Meyers *et al.*, 1976). Here it has to be kept in mind that, in the *in vitro* assay, the same number of lymphocytes is always used for testing, independent of the volume of blood needed to yield this number of cells. *In vivo*, however, the highly significant decrease of the absolute number of peripheral blood T cells in group CD 2 might influence the skin response to DNCB, as also indicated by the extreme decrease of the calculated '*in vivo* correlate' in form of the blood response capacity (BRC) in group CD 2. The same clinical parameters which were demonstrated to influence, though considerably less pronounced, the *in vitro* primary CMI also accounted for the deficiency of the blood response capacity in CD 2, and in addition also for three of the four CD 1 patients, who exhibited an extremely low BRC. In accordance with these conclusions would be the observation by Meyers *et al.* (1976) who reported a high DNCB anergy rate in Crohn's disease patients, most of whom had a very long duration of disease and active disease, the latter being the case also in the two patients with short-standing disease, who were DNCB anergic (Meyers, S., personal communication). Similarly, Bolton *et al.* (1974) found an impaired DNCB responsiveness only in highly active Crohn's disease patients who were simultaneously on steroids.

The data presented may allow the conclusions that, in the *in vitro* primary CMI, the lymphocyte *per se* as well as the function of accessory cells—for example, macrophages—as to the extent necessary to support the MLC is equally reactive in Crohn's disease as in normal subjects and in patients with other inflammatory bowel disease. Deviations from normal are secondary to disease parameters—for example, duration, activity, and, possibly, long-lasting derangements due to immunosuppressive drugs.

In tests in which Crohn's disease patients were stimulated by other CD patients, there was no clear indication for a similarly low responsiveness as

described for patients with rheumatoid arthritis (Stastny, 1976), which was due to the sharing of genetically controlled LD-determinants in these patients. However, the number of those tests was small in this study, so this point awaits further clarification.

The significantly lower stimulatory capacity in highly active CD 1 patients than in inactive CD 1 patients is in agreement with the significantly lower than normal stimulatory capacity of the patients of group D. This relation of the stimulatory capacity to the CDAI, however, was evident only in CD 1 not in CD 2, which indicates that additional influences appear as the disease progresses or that this significant difference in CD 1 is purely coincidental. Whatever the causes are, the reduction of the stimulatory capacity of PBL in Crohn's disease is secondary to the disease and thus has little bearing on the aetiology of CD.

The authors wish to express their gratitude to Dr H. Malchow, Dr H. Sommer, and Dr H. Ehms for support and for allowing them to study their patients, and Dr A. Schimpl and Dr E. Wecker for stimulating discussion.

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