K-lymphocytes (killer-cells) in Crohn's disease and acute virus B-hepatitis¹

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SUMMARY Total lymphocyte counts, B-, T-, C'3 receptor-bearing lymphocytes, and K-cell activity were studied in peripheral blood in patients with Crohn's disease and inflammatory liver disease. Patients with active untreated Crohn's disease and acute virus B hepatitis exhibited a markedly increased K-cell activity measured in a plaque assay when compared with normal controls ($p < 0.01$). Patients with immunosuppressive treated Crohn's disease, HB_sAg-positive chronic active hepatitis, and cirrhosis of the liver showed only a slight increase of K-cell activity ($p < 0.01$). In the post-acute phase of hepatitis (four to 12 weeks from onset) K-cell activity fell to normal levels. The number of B-lymphocytes showed a relative and absolute decrease in all groups of patients. With the exception of patients with acute HB_sAg-positive hepatitis and the post-acute phase of hepatitis all the other groups showed statistically decreased absolute numbers for C'3 receptor-bearing lymphocytes. The significant decrease in K-cell activity and the number of T-lymphocytes in Crohn's disease treated with immunosuppressive drugs was interpreted as an effect of azathioprine and prednisone on these lymphocyte subpopulations.

Patients with Crohn's disease and ulcerative colitis exhibit humoral (Deodhar *et al.*, 1969; Thayer *et al.*, 1969) and cellular immunity against intestinal antigens (Bendixen, 1967; Bendixen, 1969; Richens *et al.*, 1974; Eckhardt *et al.*, 1976) and common antigen (Thayer *et al.*, 1969; Bull and Ignaczak, 1973); the latter is an enterobacterial surface antigen, showing cross-reaction with intestinal epithelial cells (Perlmann and Hammarström, 1965; Perlmann *et al.*, 1967). It is not known at present whether K-lymphocytes play a pathogenic role in inflammatory bowel disease. This lymphocyte subpopulation is able to lyse target cells through Ig G antibodies (MacLennan and Harding, 1970; Möller and Svehag, 1972; Perlmann *et al.*, 1972; Forman and Möller, 1973). Previous studies have been concerned with K-cell activity in autoimmune thyroid disease, systemic lupus erythematosus, polyarteritis

nodosa, Sjögren's syndrome, rheumatoid arthritis, chronic active hepatitis, and Hodgkin's disease (Holm *et al.*, 1973; Schneider *et al.*, 1975; Wisloff *et al.*, 1975; Cochrane *et al.*, 1976; Calder *et al.*, 1976; Feldmann *et al.*, 1976).

The present study is concerned with the determination of K-cell activity and the measurement of B-, T-, and C'3 receptor-bearing lymphocytes in the peripheral blood of patients with Crohn's disease. Normal individuals and patients with inflammatory liver disease served as controls. Inflammatory liver diseases—for example, acute virus B-hepatitis and HB_sAg-positive chronic active hepatitis (CAH)—were selected for comparison as *in vivo* membrane-fixed Ig G was demonstrated on the hepatocytes (Hopf *et al.*, 1975; Meyer zum Büschenfelde *et al.*, 1976). These findings suggest that antibody- or immune complex-mediated cytotoxic reactions have to be considered in the pathogenesis of these diseases.

Methods

PATIENTS

Seventy-one patients with Crohn's disease in an acute

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phase without therapy ($n = 21$) and in a chronic phase under immunosuppressive therapy with azathioprine and prednisone ($n = 50$), fifty patients with HB_sAg-positive hepatitis (31 patients in an acute phase, one to two weeks from onset; 19 patients in the postacute phase, four to 12 weeks from onset), 21 patients with HB_sAg-positive CAH, and 25 patients with cirrhosis of the liver from various causes were investigated. None of the patients with liver diseases received immunosuppressive drugs. Forty normal individuals served as controls.

K-CELLS

K-cell activity of lymphocytes isolated from the peripheral blood was determined by the plaque assay described by Biberfeld *et al.* (1975):

Chicken erythrocytes

These (nick chick, HNL, Dieburg, Germany, aged 10-16 weeks) served as target cells; the experiments were performed with a 5% erythrocyte suspension in Hanks' medium.

Anti-chicken red cell serum

This was obtained by immunisation of rabbits (weekly 0.5 ml packed red cells in 0.15 M NaCl, intravenously over five weeks). Preliminary experiments showed that the highest number of plaques was obtained at a 1:2500 dilution of the heat-inactivated serum in RPMI 1640 + 15% FCS (heat-inactivated) + 0.2 mMol Hepes buffer (pH 7.4). All experiments were performed with a single rabbit anti-chicken red cell antibody.

Human lymphocytes

These were isolated from heparinised peripheral blood by the method of Kissmeyer-Nielsen and Kjerbye (1967): addition of 300 mg iron powder per 10 ml blood (Merck, Darmstadt, Germany), incubation for 10 minutes at 37°C in a water bath. The lymphocytes were separated on a Ficoll-Isopaque gradient after sedimentation of erythrocytes in 5% dextran (MW 200 000) for 30 minutes at 37°C and washed three times in Hanks' medium. Lymphocytes were resuspended in RPMI/FCS medium at a concentration of 1×10^6 cells/ml. Monocytes were identified by specific staining for their endogenous peroxidase activity (Preud'Homme and Flandrin, 1974), α -naphthylacetate-esterase activity, and by their capacities to phagocytose latex particles. According to these criteria the lymphocyte suspensions contained less than 1% monocytes.

Performance of plaque assay

Petri dishes (35 $\times$ 10 mm, Falcon Plastics) were

treated with a solution of 50 μ g poly-L-lysine (MW 100 000, Serva Feinbiochemica, Heidelberg, Germany) in 1 ml Hanks' medium for 45 minutes, washed with Hanks' medium and incubated with 1 ml of a 5% chicken erythrocyte suspension (see above) for 45 minutes at room temperature. After non-adherent cells have been removed by washing, a homogeneous erythrocyte monolayer is obtained. One millilitre of anti-chicken red cell serum (see above) and 1 ml lymphocyte suspension (see above) were added and the petri dishes incubated for 20 hours at 37°C in a CO₂ gassed incubator. An area corresponding to ≥ 5 lysed erythrocytes was defined as a plaque. The plaques were counted under a microscope ($\times 320$ magnification) after fixation with 2.5% glutaraldehyde. The mean value from 20 different fields per test was used for further calculations.

The following controls were carried out simultaneously:

1. Monolayer + lymphocyte suspension (no anti-chicken red cell serum); this control did not show plaque formation.
2. Monolayer + anti-chicken red cell serum (no lymphocyte suspension); this control showed $\bar{x} = 0.5 \pm 0.2$ plaques, possibly induced by a low complement activity in the medium after inactivation.

B-LYMPHOCYTES

Peripheral blood lymphocytes were separated as described above. 1×10^6 lymphocytes were incubated for 30 minutes at 37°C, washed three times at 37°C in TC medium, and stained at 4°C for 30 minutes with FITC-conjugated sheep anti-human-Ig A, M, G (Behring-Werke, Marburg, Germany).

T-LYMPHOCYTES

One millilitre of a 5% suspension of sheep erythrocytes was incubated for 60 minutes at 37°C in TC medium with 0.2 ml of a neuraminidase solution (1 unit/ml, Behring-Werke, Marburg, Germany). After three washes a 0.5% erythrocyte suspension in TC medium was prepared.

One $\times 10^6$ peripheral blood lymphocytes (preparation see above) were incubated with 0.5 ml of the 0.5% neuraminidase-treated sheep red cell suspension for 10 minutes at 37°C and centrifuged at 1000 rpm. The cell pellet was placed at 4°C for 60 minutes and then carefully resuspended.

C'3 RECEPTOR-BEARING LYMPHOCYTES

One volume of sensitised sheep erythrocytes (5×10^8 /ml), treated with 0.06% glutaraldehyde, was incubated for 30 minutes at 37°C with one volume of 1:2 diluted fresh human serum, washed three times and adjusted to 5×10^7 /ml and used in the

rosette assay to detect complement receptor-bearing lymphocytes. Fifty μl of EAC-suspension ($5 \times 10^7/\text{ml}$ TC medium) were incubated with 50 μl of a human lymphocyte suspension ($1 \times 10^6/\text{ml}$ TC medium, separation see above) for 30 minutes at 37°C . The total number of rosettes per 200 lymphocytes was assigned as the percentage of C'3 receptor-bearing lymphocytes.

TOTAL NUMBER OF LYMPHOCYTES

Concentration of leucocytes was measured in a coulter counter and the total number of lymphocytes calculated by means of a differential blood count.

STATISTICS

The values of the control group and patient groups were compared by means of the non-parametric U-test of Mann-Whitney-Wilcoxon.

Results

The results are summarised in the Figure and Tables 1 and 2.

TOTAL LYMPHOCYTE COUNT

The absolute count of lymphocytes was increased significantly in patients with acute virus B-hepatitis ($P < 0.05$), whereas there was a decrease in patients with immunosuppressive treated Crohn's disease, HB_sAg-positive CAH, and cirrhosis of the liver when compared with normal controls ($P < 0.05$ and < 0.01 respectively, Table 1). Patients with postacute virus B-hepatitis and active untreated Crohn's disease did not differ from normal controls ($P > 0.05$, Table 1).

B-LYMPHOCYTES

The relative and absolute B-lymphocyte counts in the control group were 8% and $0.14 \times 10^3/\text{mm}^3$ respectively. Patients with active untreated Crohn's disease, immunosuppressive treated Crohn's disease, acute HB_sAg-positive hepatitis, postacute virus B-hepatitis (four to 12 weeks from onset), HB_sAg-positive CAH, and cirrhosis of the liver showed a relative and absolute decrease of B-lymphocyte counts. The statistical comparison revealed signi-

Figure Number of plaques per field in the plaque assay in normal controls, patients with Crohn's disease, and inflammatory liver disease. The medians (m) are shown.

Table 1 Peripheral blood lymphocyte subpopulations in normal controls, patients with Crohn's disease, and inflammatory liver disease

	Lymphocytes		B-lymphocytes		T-lymphocytes		K-cells
	No.	Absolute*	%	Absolute*	%	Absolute*	Plaques†
Controls	40	1.93	8	0.14	66	1.29	3.90
Hepatitis (acute) HB _s Ag-positive	31	2.20 P < 0.05	2 P < 0.01	0.05 P < 0.01	63 NS	1.45 NS	9.0 P < 0.01
Hepatitis (post-acute)	19	1.84 NS	3 P < 0.01	0.06 P < 0.01	66 NS	1.18 NS	4.40 NS
Crohn's disease No immunosuppression	21	2.0 NS	3 P < 0.01	0.06 P < 0.01	65 NS	0.94 NS	10.30 P < 0.01
Immunosuppression	50	1.48 P < 0.05	4 P < 0.01	0.07 P < 0.01	62 NS	0.88 P < 0.01	6.85 P < 0.01
Chronic active hepatitis, HB _s Ag-positive	21	1.40 P < 0.05	3 P < 0.01	0.04 P < 0.01	54 P < 0.05	0.74 P < 0.01	5.70 P < 0.01
Cirrhosis of liver	25	1.12 P < 0.01	3 P < 0.01	0.05 P < 0.01	59 P < 0.05	0.68 NS	5.50 P < 0.01

The medians are shown, which were calculated independently in each patient group. * $\times 10^3/\text{mm}^3$. † number of plaques for field. NS = not statistically significant in Mann-Whitney-Wilcoxon's U-test ($P > 0.05$).

Table 2 Peripheral blood lymphocytes bearing C'3 receptors in normal controls, patients with Crohn's disease, and inflammatory liver disease. The medians are shown.

	No.	%	Absolute*
Controls	41	12	0.26
Hepatitis (acute) HB _s Ag-positive	15	8 P < 0.05	0.19 NS
Hepatitis (post-acute)	12	7 P < 0.01	0.21 NS
Crohn's disease No immunosuppression	16	9 P < 0.01	0.16 P < 0.05
Immunosuppression	45	9 P < 0.01	0.11 P < 0.01
Chronic active hepatitis, HB _s Ag-positive	8	7 P < 0.01	0.10 P < 0.01
Cirrhosis of liver	19	8 P < 0.05	0.12 P < 0.05

* $\times 10^3/\text{mm}^3$.

NS = not statistically significant in Mann-Whitney-Wilcoxon's U-test ($P > 0.05$).

ficant difference between the control group and the various patient groups ($P < 0.01$, Table 1).

T-LYMPHOCYTES

The relative and absolute T-lymphocyte counts in the control group were 66% and $1.29 \times 10^3/\text{mm}^3$ respectively. The relative number of T-lymphocytes did not differ between patients and controls, with the exception of decreased values in the groups with HB_sAg-positive CAH and cirrhosis of the liver

($P < 0.05$, Table 1). The absolute number, however, showed a significantly lower count of T-cells in patients with immunosuppressive treated Crohn's disease and HB_sAg-positive CAH when compared with normal controls ($P < 0.01$, Table 1). The results in patients with active untreated Crohn's disease, acute HB_sAg-positive hepatitis, postacute virus B-hepatitis, and cirrhosis of the liver did not differ from normal controls ($P > 0.05$, Table 1).

K-LYMPHOCYTES

Patients with Crohn's disease, acute HB_sAg-positive hepatitis, HB_sAg-positive CAH, and cirrhosis of the liver showed a significantly higher K-cell activity compared with normal controls ($P < 0.01$, Table 1). The highest numbers of plaques were observed in patients with acute HB_sAg-positive hepatitis one to two weeks from onset (9 plaques/field) and patients with active untreated Crohn's disease (10.3 plaques/field) as opposed to 3.9 plaques/field in normal controls. Patients with immunosuppressive treated Crohn's disease, HB_sAg-positive CAH, and cirrhosis of the liver had only a slightly increased K-cell activity ($P < 0.01$, Table 1). In the postacute phase of hepatitis (four to 12 weeks from onset) there was a decrease of K-cell activity (4.40 plaques/field) to normal values ($P > 0.05$).

When K-cell activity was compared in the various groups of patients significant differences were noted between active untreated Crohn's disease and immunosuppressive treated Crohn's disease, between acute virus B-hepatitis (one to two weeks from onset) and HB_sAg-positive CAH as well as the post-acute phase (four to 12 weeks from onset, $P < 0.01$).

In contrast, the K-cell activity in acute HB_sAg-positive hepatitis and active untreated Crohn's disease was not statistically different ($P > 0.05$).

C'3 RECEPTOR-BEARING LYMPHOCYTES (Table 2)

The relative and absolute values of C'3 receptor-bearing lymphocytes in the control group were 12% and $0.26 \times 10^3/\text{mm}^3$ respectively. Patients with Crohn's disease, HB_sAg-positive CAH, and cirrhosis of the liver showed significantly decreased relative and absolute numbers ($P < 0.05$ and < 0.01 respectively), but there was only a relative decrease in the patient groups with acute HB_sAg-positive hepatitis (one or two weeks from onset) and post-acute phase of hepatitis (four to 12 weeks from onset).

Discussion

The plaque assay had been used previously for characterisation of antibody-dependent lymphocyte cytotoxicity at the single cell level. Furthermore, the assay seems to be feasible for K-cell measurement. This test system, introduced by Biberfeld *et al.* (1975), uses sheep and young chicken or bovine erythrocytes (Wählin *et al.*, 1976) as target cells. It has been shown that in man, in addition to K-lymphocytes, polymorphonuclear leucocytes and blood monocytes are also able to kill antibody-coated erythrocytes (Perlmann and Perlmann, 1970; Holm, 1972; Perlmann *et al.*, 1972; Holm and Hammarström, 1973; Papamichail and Temple, 1975; Trinchieri *et al.*, 1975). By using this target cell system it is therefore important to remove these phagocytic effector cell types quantitatively. Our experiments showed that, after treatment with iron powder, the lymphocyte suspensions contained less than 1% monocytes and polymorphonuclear leucocytes, while without pretreatment the proportion of monocytes was between approximately 20-30%, leading to an increase of plaque numbers of approximately 20-30%. Preliminary experiments had shown that the number of plaques increased proportionally with the number of added lymphocytes and with the incubation time. Optimal test conditions were obtained with a 20-hour incubation and 1.2×10^6 lymphocytes per petri dish. In addition, the number of plaques was dependent on the concentration of the anti-chicken red cell serum. When antibody was present in the test medium optimal plaque numbers were obtained only in a narrow zone of antibody concentration. This has to be determined for each individual antiserum. The plaque assay, as used in this study, was highly reproducible.

Our investigations show a high K-cell activity in

patients with active untreated Crohn's disease and acute HB_sAg-positive hepatitis. In addition to a T-cell mediated immunity against intestinal antigens and common antigen, an antibody-dependent cell-mediated cytotoxicity (ADCC) in Crohn's disease has to be discussed. The antigen specificity of this immunological reaction in Crohn's disease requires further study. The characterisation of immune complexes in Crohn's disease (Jewell and MacLennan, 1973) may be important in this respect. Studies in our laboratory show that these immune complexes contain no common antigen (Eckhardt *et al.*, in preparation).

The statistical analysis revealed no differences in the high K-cell activity between acute HB_sAg-positive hepatitis and active untreated Crohn's disease. With the recently discussed viral aetiology in Crohn's disease (Beeken *et al.*, 1976) it would be necessary to investigate whether other viral infections also demonstrate high K-cell activity. It is known that sera with antibodies against Epstein-Barr virus (EBV) or mumps induce an ADCC against EBV or mumps virus-superinfected target cells (Jondal, 1976; Härfast *et al.*, 1975).

Our studies have demonstrated that the total number of lymphocytes in active untreated Crohn's disease is normal, whereas patients with acute virus B hepatitis showed increased values. The groups of patients with immunosuppressive treated Crohn's disease, HB_sAg-positive CAH, and cirrhosis of the liver demonstrated decreased counts. In addition, we could show that patients with active untreated Crohn's disease and acute HB_sAg-positive hepatitis have a normal proportion of T-lymphocytes in the peripheral blood. This finding is in accordance with the results of Bird and Britton (1974) and Thayer *et al.* (1975) in Crohn's disease. In contrast with our results Edgington and Chisari (1975) and Strickland *et al.* (1974) found decreased T-lymphocyte numbers in virus B hepatitis and Crohn's disease. The accumulated evidence shows no definite proof of an impairment of T-lymphocyte function in Crohn's disease (Whorwell and Wright, 1976). The demonstration of cellular immune reactions against intestinal antigens in the leucocyte migration inhibition test (Richens *et al.*, 1974; Eckhardt *et al.*, 1976) rather suggests an unimpaired function of these lymphocyte population in Crohn's disease.

Recent studies of Lobo *et al.* (1975) indicated two populations of Ig-bearing lymphocytes in healthy subjects: one with surface stable Ig determinants (preincubation and washing of the cells at 37°C before staining, mean value 9%), and another that lacks these markers but has receptors capable of binding exogenous Ig G (mean value 22%). The determination of B-lymphocytes in the present study

was performed after preincubation at 37°C. The proportion of B-lymphocytes in the control group in our study is similar to the one reported by Lobo *et al.* (1975) for surface stable Ig determinants. The decrease of peripheral B-lymphocytes in patients with Crohn's disease and inflammatory liver disease is in accordance with recent studies in patients with Crohn's disease by Høj and Sørensen (1976). It is not known whether the decrease of B-lymphocytes is correlated with an increase in K-cell activity.

There is evidence that B-lymphocytes carry a receptor for activated C'3 (Jondal *et al.*, 1973). In contrast with the decrease of peripheral blood B-lymphocytes, Table 2 demonstrates no statistically significant difference between the absolute numbers of C'3 receptor-bearing lymphocytes in normal controls and patients with acute HB_sAg-positive hepatitis, whereas the groups with Crohn's disease, HB_sAg-positive, CAH, and cirrhosis of the liver exhibited decreased values. The differences between B-cell and C'3 receptor-bearing cell numbers in each individual case of all patient groups showed no correlation with the K-cell numbers. Although the findings of Perlmann *et al.* (1972) and Biberfeld *et al.* (1975) show that K-cells carry a receptor for activated C'3 this cannot be deduced from our data.

The significant decrease of peripheral blood T-lymphocytes and of K-cell activity in patients with Crohn's disease under immunosuppressive therapy as compared with normal controls has to be interpreted as a specific effect of azathioprine and prednisone on these lymphocyte subpopulations. Similar results have been reported by Campbell *et al.* (1974), Röllinghof *et al.* (1973) concerning the effect of azathioprine on K- and T-lymphocytes and by Balow *et al.* (1975) concerning a long-term effect of glucocorticosteroids on cell-mediated immunity.

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