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Elevated numbers of peripheral T cells in inflammatory bowel diseases displaying T9 antigen and Fcα receptors

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SUMMARY

Elevated numbers of peripheral T cells expressing the activation associated antigen T9 are found in patients with active Crohn's disease. Expression of T9 is found to be correlated to the activity of the disease. However the presence of activated peripheral T cells is not restricted to Crohn's disease, but could also be found in other maladies with a supposed involvement of the immune system, e.g. ulcerative colitis, sarcoidosis, connective tissue disease and after organ transplantation. Significant elevation of the number of activated T cells could not be detected in cases of viral or bacterial enteritis and coeliac disease. Analysing the subset of T9 positive T cells with regard to the expression of Fcα receptors, a significantly increased number of Fcα receptor positive cells, within the subset of T9 positive cells in the peripheral blood of patients with Crohn's disease and ulcerative colitis was found, which could not be demonstrated in the case of other diseases analysed in this study. Thus the T9+ Fcα receptor + T cell subset may be considered to be pathognomonic for inflammatory bowel diseases. Analysis of the regulatory properties of T9 positive cells, with regard to the immunoglobulin isotype secretion in a pokeweed mitogen stimulated autologeous B cell assay, suggests that peripheral T9 positive T cells are involved in the suppression of IgA synthesis or secretion.

Keywords Crohn's disease T9 antigen Fcα receptor

INTRODUCTION

Inflammatory bowel diseases are characterized by recurrent exacerbations of clinical symptoms as is the case in many other maladies with a presumed immunological background. In an attempt to measure the clinical activity of the disease, an index was established based on the patient's own observations, the observations of his doctor, and some general parameters believed to reflect the extent of inflammation (Best *et al.*, 1976). Furthermore attempts have been made to correlate the increase of some acute phase reactants with the clinical course of inflammatory bowel diseases (Cox, Fowler & Cox, 1958; André *et al.*, 1981; Fagan *et al.*, 1982; Karbach, Ewe & Bodenstein, 1983). However none of these seems to play a role in the pathogenesis of the disease.

In previous studies we have shown that in patients with Crohn's disease elevated numbers of peripheral T cells could be detected expressing activation associated antigens on their cell surface, for example the T9 antigen (Raedler *et al.*, 1985). The differences between patients with active Crohn's disease and patients in remission or control subjects, were highly significant ($P = 0{\cdot}0001$). The number of activated T cells was correlated with the activity of the disease as expressed in the Crohn's disease activity index. However elevated numbers of activated peripheral T cells could also

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be found in other diseases believed to involve the immune system (Raedler *et al.*, 1984; Yu *et al.*, 1980). Starting from the hypothesis that the T cell subset which possesses activation associated antigens plays a role in the immunopathogenesis of Crohn's disease, we characterized these cells with regard to further cell surface determinants and their functional properties. The following experiments were designed to answer the question as to whether activated T cells from Crohn's disease are distinguishable from those found in increased number in other diseases (e.g. ulcerative colitis, sarcoidosis, connective tissue diseases, rheumatoid arthritis, after organ transplantation). Evidence was also sought as to the possible involvement of this cell subset in the immune response of Crohn's disease.

MATERIALS AND METHODS

Patients and controls. Hospitalized patients with active Crohn's disease ($n=40$), with ulcerative colitis ($n=10$), sarcoidosis ($n=8$), lupus erythematodes ($n=6$), rheumatoid arthritis ($n=5$), after heart, liver and kidney transplantation ($n=4$), viral and bacterial enteritis ($n=11$) and pseudomembranaceous colitis ($n=1$) as well as coeliac disease ($n=6$) were examined.

Patients with Crohn's disease had >150 in the Crohn's disease activity index. Activity of ulcerative colitis was confirmed endoscopically and histologically. Eight of the patients with Crohn's disease had involvement of the large bowel. All patients with ulcerative colitis showed an inflammation of the mucosa predominantly in the distal part of the large bowel. Of the patients with active Crohn's disease ($n=18$) 15 were being treated with moderate doses of steroids (12–40 mg prednisone). Nine of these patients were analysed for T9 expressing cells before steroid treatment. Patients with Crohn's disease not restricted to the small intestine additionally received sulphasalazine. All patients with ulcerative colitis were treated with sulphasalazine, seven patients additionally received steroids, five of which were analysed before and after steroid administration. Patients undergoing allotransplantation were treated with steroids and cyclosporine A.

Preparation of lymphocytes. Samples of 10 ml blood were taken and added to 10 ml of 0·9% saline supplemented with 2,000 units of heparin. Two and a half millilitres of 'Lymphoprep' (Nyegaard) were overlayered with 5·0 ml of diluted blood and centrifuged for 30 min (20°C, 560*g*). The lymphocytes were recovered from the interphase and washed twice with phosphate-buffered saline (GIBCO). The pellet was suspended in 15 ml of cell culture medium (RPMI 1640, GIBCO) supplemented with 10% fetal calf serum (Medac) and cultured for 2 h at 37°C, for removal of monocytes. Non-adherent cells were then collected and layered on Petri dishes (05·5 cm, 5×10^6 cells per plate) coated with goat anti-human immunoglobulin (Tago, 4 ml, 10 μl/ml). After incubation for 60 min at room temperature non-adherent (non-surface immunoglobulin bearing) cells were recovered.

Controls of this separation step were performed by labelling non-adherent cells with goat anti-human Ig $(Fab)_2$ antibodies (1:20, Tago) and anti-Leu 12 (1:20, Becton–Dickinson) and adherent ones with T3 (5 μl/10^6 cells).

Enumeration of T9 positive cells. One million of the non-glass adherent, non-surface immunoglobulin bearing cells were incubated with 5 μl of T9 (Ortho-mune). Controls were carried out through the omission of the monoclonal antibody (MoAb) or alternatively through the use of normal mouse serum. Cells were incubated for 30 min at 4°C and afterwards washed three times in culture medium. Pellets were then suspended in 50 μl of rhodamine coupled goat $(Fab)_2$ anti-mouse IgG (Tago) diluted 1:60 with PBS (30 min, 4°C) and subsequently washed three times with culture medium. Cells were then resuspended, examined and counted (2–3 $\times$ 100 cells) under a fluorescence microscope (Zeiss). Microscopic counting of fluorescentic cells was controlled by flow cytometry analysis.

Enumeration of Fcα receptor+cells within the T9$^+$ subset. Non-glass adherent, non-surface immunoglobulin bearing cells were incubated in T9 (1×10^7 in 50 μl) and layered on Petri dishes, which were previously coated with goat anti-mouse IgG (Tago, 4 ml), 10 μl/ml). After incubation for 60 min at room temperature the supernatants were carefully decanted and the non-adherent cells collected. The Petri dishes, containing the adherent cells were overlayered with culture medium and

stored for 24 h for loosening and recovering. Samples of non-adherent and adherent cells were labelled with T9 as described above to control the efficiency of separation. Adherent and non-adherent cells were then given on Petri dishes coated with goat anti-human $(Fab)_2$ immunoglobulin (4 ml, 15 μl/ml, Medac) in a first step and with heat-aggregated human IgA (4 ml, 10 μl/ml, Behring) in a second step (60 min, room temperature). Adherent and non-adherent cells were counted. Adherent and non-adherent cells of the first panning step ($T9^+/T9^-$) and the second panning step (FcR^+/FcR^-) were additionally incubated in biotinylized heat-aggregated human IgA (30 min, room temperature) after which they were suspended in avidin–FITC (Medac, 1:10, 30 min, room temperature). Fcα panning was additionally controlled by a second Fcα panning resulting in less than 2% adherence of cells that were non-adherent in the first Fcα panning step. Moreover Fcα panning was completely inhibited in the presence of soluble human IgA added to the cell suspension.

Enumeration of T9 antigen on Fcα receptor$^+$ T cells. In these experiments non-glass adherent, non-surface immunoglobulin bearing cells were first given on Petri dishes coated with heat-aggregated human IgA (see above), adherent and non-adherent cells harvested and incubated in T9 and goat $(Fab)_2$ anti-mouse IgG subsequently (see above).

Autologeous pokeweed mitogen (PWM) stimulated B cell assay. Surface immunoglobulin bearing cells were separated using direct panning through goat anti-human immunoglobulin coated Petri dishes from the pool of non-glass adherent lymphocytes. One million cells were given in culture medium (RPMI 1640, Gibco, plus 10% fetal calf serum and 50 μg/ml PWM, Gibco and antibiotics) and cultured for 5 days. One million $T9^+$ or $T9^-$ cells (prepared by panning as described above) or no T cells at all were added to the B cell cultures. After 5 days the supernatants were collected and analysed for IgG, IgM and IgA isotypes by an enzyme linked immunoadsorbent assay using goat anti-human IgG, M or A (Medac) and an alkaline phosphatase coupled antibody (Medac), respectively.

RESULTS

Assessment of $T9^+$ T cells

As is shown in Fig. 1, determination of activation associated antigens on peripheral T cells in patients suffering from active Crohn's disease and ulcerative colitis showed that high numbers of peripheral T cells exhibited T9 on their cell surface. Similar results were obtained in patients with connective tissue diseases and sarcoidosis during acute exacerbation (stage II–III). Elevated numbers of $T9^+$ cells could also be detected in recipients of transplants. In contrast patients with a viral and bacterial enteritis as well as pseudomembranaceous colitis had only low levels of T9

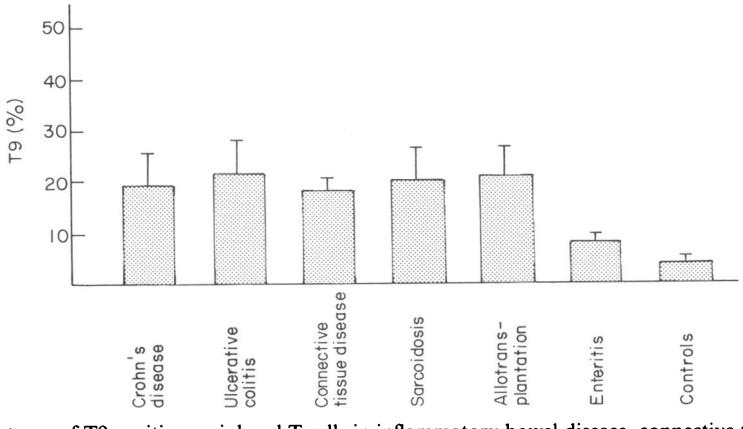

Fig. 1. Percentages of T9 positive peripheral T cells in inflammatory bowel disease, connective tissue disease, sarcoidosis, acute enteritis and after allotransplantation.

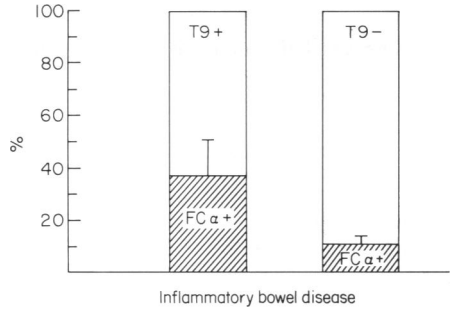

Fig. 2. Percentages of Fcα receptor positive cells within the subsets of T9 positive and T9 negative T cells in patients with Crohn's disease and ulcerative colitis ($n=8$).

expressing T cells if analysed during the first or second week of hospitalization. Two patients with salmonellosis were examined at the onset of the disease before treatment with antibiotics. Most interestingly only 1–2% of peripheral T cells were positive for T9. Activated $T9^+$ T cells of patients with acute exacerbation of coeliac disease ($n=2$) or in remission ($n=4$) were within the normal range (6·1%, s.d. 1·3). In patients with inflammatory bowel disease, there was no difference in T9 expression between those treated with steroids and those not. Moreover there was no significant difference in the number of activated T cells before and after administration of steroid and non-steroid drugs.

Assessment of Fcα receptor positive and negative T cells within the subsets of $T9^+$ and $T9^-$ cells
Separation of $T9^+$ T cells from $T9^-$ T cells, and subsequent determination of Fcα receptor bearing cells within both subsets, showed that within the $T9^+$ population, Fcα receptor positive cells are significantly increased, if compared with the population of Fcα receptor negative T cells within the subset of T9 negative cells in patients with Crohn's disease and ulcerative colitis (Fig. 2).

Assessment of T9 positive and negative cells within the subsets of Fcα receptor positive and negative T cells
Analysis of T9 positive T cells within the subset of Fcα receptor positive or negative T cells in patients with inflammatory bowel diseases showed that a significant number of the Fcα receptor positive cells are also positive for T9, whereas in the Fcα receptor negative subset the rate of $T9^+$ cells was low (Fig. 3). In patients with connective tissue diseases there were low numbers of T9 positive cells within the subset of Fcα receptor positive ones, whereas the majority of T9 positive T cells could be found in the population of Fcα receptor negative ones. A similar relationship could be found in sarcoidosis and in recipients of allotransplants. Comparison of Crohn's disease with

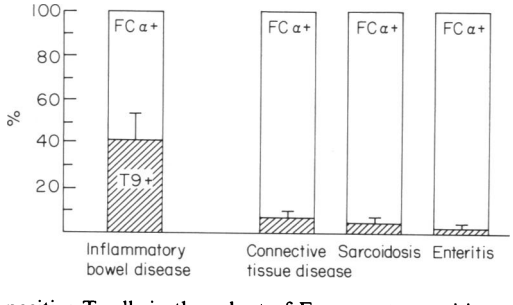

Fig. 3. Percentages of T9 positive T cells in the subset of Fcα receptor positive cells in inflammatory bowel disease, connective tissue disease, sarcoidosis and acute enteritis.

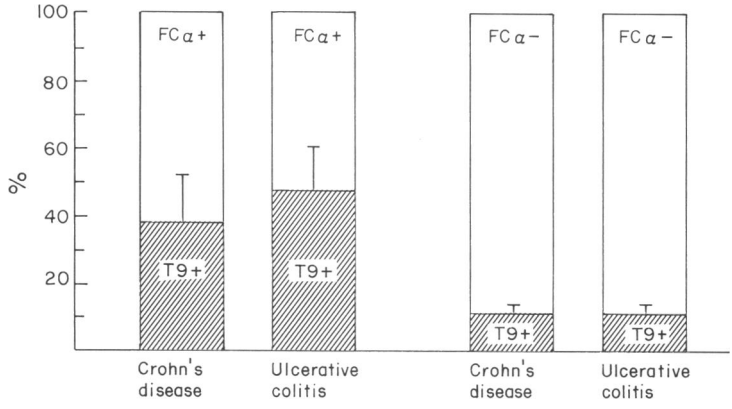

Fig. 4. Percentages of T9 positive T cells within the subset of Fcα receptor positive and negative cells in patients with Crohn's disease and ulcerative colitis.

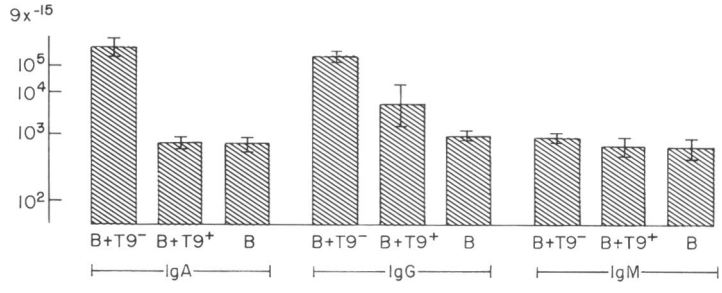

Fig. 5. IgA, IgG and IgM in the supernatants of PWM stimulated autologeous B cell cultures with or without addition of T cell subpopulations.

ulcerative colitis showed that the population of Fcα receptor positive, T9 positive T cells is elevated significantly in both cases (Fig. 4).

Regulatory properties of T9⁺ and T9⁻ cells in an autologeous B cell assay in patients with Crohn's disease

Comparison of immunoglobulin production in a autologeous PWM stimulated B cell assay between T9 positive and T9 negative T cells added to B cells in a ratio of 1:1 or immunoglobulin production in pure B cell cultures revealed that in the case of IgM no significant differences could be observed. In contrast the IgA production is significantly elevated if T9 positive cells are eliminated (Fig. 5). The IgG production is influenced in a lesser degree by the elimination of T9 positive cells. However in the presence of T9 positive T cells, significantly more IgG is detectable in the supernatants than in case of cell cultures containing B cells only.

DISCUSSION

Activation of T cells by, for example, antigen presenting cells is thought to be accompanied by several differentiation processes, among them the blast transformation of activated cells and the expression of cell surface determinants on their cell surface (Yu *et al.*, 1980; Hercend *et al.*, 1981). Whereas the enumeration of helper, suppressor, or total number of peripheral T cells in patients with Crohn's disease has failed to reveal any striking differences between patients with active disease and normal controls (Selby & Jewel, 1983; Pepys *et al.*, 1982; Richens *et al.*, 1980), assessment of

activated T cells is correlated with the activity of Crohn's disease (Raedler *et al.*, 1984). Thus clinical improvement of patients during treatment is accompanied by a decrease in the number of activated T cells exhibiting the T9 and the HLA/DR antigen. Flow cytometry experiments revealed that the appearance of these determinants is restricted to T cells of large size, in contrast to medium sized and small ones which express only low amounts or none of these antigens (Raedler *et al.*, 1984). Of the activation associated antigens examined in previous studies, e.g. the T9 antigen and the HLA/DR antigen, the former has been preferred in the present experiments. It has been shown that the T9 subset exceeds the HLA/DR T cell subset, and also that all HLA/DR$^+$ T cells are T9$^+$ (Raedler *et al.*, 1984). This may be explained by the results of *in vitro* experiments showing that T9 expression precedes the expression of HLA/DR after activation (Hercend *et al.*, 1981). A further disadvantage in the use of HLA/DR is that many B cells express this antigen. The T9 antigen defined by the T9 MoAb is thought to be identical with the transferrin receptor (Goding & Burns, 1981; Sutherland *et al.*, 1981). Its expression is thus not restricted to cells of the immune system but seems to be detectable on proliferating cells in general, for example after malignant transformation. After removal of monocytes and B cells, T cells are the only cell type in the peripheral blood exhibiting increased amounts of the T9 antigen in patients with Crohn's disease, ulcerative colitis, sarcoidosis, connective tissue diseases and after organ transplantation as well. Thus assessment of T9 may turn out to be a valuable clinical parameter for the involvement of the immune system in these maladies.

Although the subset of T9 positive T cells is not specific for the immune response in Crohn's disease, we wondered whether, within this subset, regulatory T cells could be detectable—cells that may play a role in the immunopathogenesis of Crohn's disease. The hypothesis of an immunopathogenesis of Crohn's disease is primarily based on an accumulation of immunocytes within the affected mucosa. Whereas the physiological humoral immune response of the mucosa associated immune system is mediated by IgA antibodies, IgA B cells are replaced in the affected mucosa by IgM and IgB cells (Persson & Danielsson, 1973; Baklien & Brandtzaeg, 1976; Otto & Gebbers, 1978). Moreover there are several observations that point to a disturbed regulation of the immune response especially with regard to its suppressor branch both within the mucosa and the peripheral blood in Crohn's disease (Victorino & Hodgson, 1980; Elson *et al.*, 1981; Holdstock, Chastenay & Krawitt, 1981; Fiochi, Youngman & Farmer, 1983; Auer, Röder & Frölich, 1984). Starting from the observation that the IgA response is regulated by T cells bearing a receptor for the IgA heavy chain (Endoh *et al.*, 1981) we analysed the T9 positive subset for the expression of a Fcα receptor. These experiments provided evidence for the existence of a subset of T cells in the peripheral blood of patients with Crohn's disease that is both positive for T9 and Fcα receptors. This subset could also be found in ulcerative colitis, but was not detectable in acute enteritis. In coeliac disease elevated numbers of activated T cells could not be found in the peripheral blood, confirming the recent findings of Selby *et al.* (1983). Moreover maladies examined that are supposed to involve the immune system fail to show a significant subset of cells characterized both by the T9 antigen and Fcα receptors. Thus we conclude that this subset may be pathognomonic for inflammatory bowel diseases.

A further characterization of T9 positive cells in Crohn's disease was performed by analysing their ability to regulate the synthesis and/or secretion of immunoglobulin isotypes by autologeous B cells. These experiments revealed that T9 positive T cells added to peripheral B cells in a ratio of 5:1 lead to a suppression of Ig production of all isotypes i.e. IgG, M and A. This is in accordance with observations recently made by Clancy, Cripps & Chipchase (1984). These authors claim that optimal T cell help for B cells is achieved at a ratio of 1:2. In our experiments addition of equal numbers of T9 negative cells to cultures of B cells provides stimulation of IgG and IgA production but apparently does not influence IgM-B cells. This may be due to a suboptimal ratio of T to B cells in case of IgM-B cells being more infrequent in the peripheral blood. In contrast T9 positive T cells do not help or even suppress help for IgA-B cells and moreover stimulation of IgG-B cells is decreased in the presence of T9 positive cells. This observation may support the hypothesis of an enhanced suppressor activity in the peripheral blood of patients with inflammatory bowel disease reported recently (Victorino & Hodgson, 1980; Elson *et al.*, 1981; Holdstock *et al.*, 1981; Fiochi *et al.*, 1983; Auer *et al.*, 1984).

Thus T9 positive cells do not only enable clinical assessment of the activity of Crohn's disease, but studies of their properties may lead to a better understanding of its pathogenesis.

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