PAPERS

Functional defect of T cells in autoimmune gastritis

J A Vargas, M Alvarez-Mon, L Manzano, A Albillos, A Fernandez-Corugedo, F Albarrán, A Duránte

Abstract

The functional response and phenotypic characterisation of peripheral blood T cells were studied in 41 patients with autoimmune gastritis – nine patients with autoimmune gastritis alone, 11 with untreated pernicious anaemia, and 21 with resolved pernicious anaemia who were taking vitamin B-12. Phenotypic analysis showed no changes in the CD4/CD8 ratio in any group of patients. CD3+ cells were significantly decreased and CD16+ cells were significantly increased in patients with autoimmune gastritis alone. Phytohaemagglutinin induced T cell proliferation, with or without interleukin 2, was reduced in the three groups. T cell proliferation induced by phorbol myristate acetate was normal. Interleukin 2 production of phytohaemagglutinin-stimulated lymphocytes was normal in the three groups. Five patients with pernicious anaemia treated with vitamin B-12 were followed and persistent hypoproliferation of T cells in response to phytohaemagglutinin was observed. The follow up study of the phenotype of these patients showed a significant increase of the CD2+ CD3– lymphocyte population after six months' treatment. In conclusion, the three groups of autoimmune gastritis patients studied have a functional defect in T cells that is independent of B-12 treatment and of the presence of pernicious anaemia.

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Keywords: T cells, autoimmune gastritis, immune system, pernicious anaemia.

Pernicious anaemia is the result of an autoimmune destruction of the gastric body mucosa. This destruction produces a lack of intrinsic factor, a product of the parietal cells that is required for the dietary absorption of vitamin B-12. The disorder is characterised histologically by gastric mucosal atrophy, selective loss of parietal and chief cells in the gastric mucosa, and submucosal lymphocytic infiltration. Autoantibodies to gastric parietal cells and to intrinsic factor are found in the circulation and in the gastric secretions.¹ There is

evidence from long term follow up studies that pernicious anaemia evolves from pre-existing type A or autoimmune chronic atrophic gastritis and these two processes can appropriately be called 'autoimmune gastritis'.^{2,3} The type of gastric lesion, the presence of autoantibodies, and the association with several autoimmune diseases and HLA types,^{1,4,5} all suggest an autoimmune aetiology, although the ultimate mechanism remains unknown.

Humoral immunity in autoimmune gastritis has been thoroughly studied, and the acid pump (H,K-ATPase) has recently been identified as the main target of gastric autoantibodies.⁶ The cellular immune response has received less attention.^{1,4,7,8} However, there is some evidence, both clinical and experimental, that cellular mechanisms may play an important part in pathogenesis.^{1,4} We aimed to carry out a phenotypic and functional analysis of T cells purified from peripheral blood. We grouped the patients according to the presence or absence of pernicious anaemia and its treatment with parenteral vitamin B-12. Five patients were studied before and after vitamin treatment.

Methods

SAMPLE

We studied 41 patients (29 women and 12 men, mean age 69.5 years) with chronic autoimmune gastritis. We divided the patients into three groups: pernicious anaemia with no treatment (11 patients); treated pernicious anaemia (21 patients); autoimmune gastritis without pernicious anaemia (nine patients). Five patients from group 1 were followed at two and six months after beginning therapy.

The diagnosis of chronic autoimmune gastritis was based on the pathological findings in gastric biopsy tissue.⁹ When this was not available (12 cases), the patient had to fulfil at least three of the following criteria: (a) compatible endoscopic image; (b) pentagastrin-fast achlorhydria; (c) hypopepsinogenaemia I and hypergastrinaemia; (d) presence of anti-parietal cell antibodies.

Diagnosis of pernicious anaemia required the presence of: (a) peripheral blood or bone marrow megaloblastosis; (b) serum cobalamin <300 pg/ml (normal range: 300–1000 pg/ml); (c) abnormal Schilling test that normalised with

Services of Internal Medicine and Gastroenterology, Department of Medicine, Clínica Puerta de Hierro, Universidad Autónoma, Madrid
J A Vargas
A Albillos
A Fernandez-Corugedo
A Duránte

Service of Internal Medicine, Department of Medicine, Hospital Universitario Príncipe de Asturias, Universidad de Alcalá de Henares, Madrid, Spain
M Alvarez-Mon
L Manzano
F Albarrán

Correspondence to: Dr J A Vargas, Service of Internal Medicine I, Clínica Puerta de Hierro, San Martín de Porres 4, 28035 Madrid, Spain.

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TABLE I Phenotypic study of purified T cells from patients with untreated pernicious anaemia, treated pernicious anaemia, and autoimmune gastritis with no pernicious anaemia, and the control group

Phenotype	Chronic autoimmune gastritis	Pernicious anaemia	Treated pernicious anaemia	Controls
CD2	90.7 (5.3)	93.4 (5.5)	91.5 (6.7)	94.8 (4.5)
CD3	62.8 (12.7)*	74.8 (6.9)	72.0 (13.0)	77.8 (14.5)
CD4	39.7 (10.9)	46.7 (10.5)	43.5 (11.2)	47.7 (14.1)
CD8	34.5 (11.7)	27.0 (10.4)	32.7 (12.6)	30.0 (10.7)
CD4/CD8	1.27 (0.31)	2 (0.9)	1.49 (0.6)	1.8 (0.8)
CD16	22.5 (9.9)*	14.2 (8.3)	16.7 (8.2)	14.0 (8.2)
CD25	2.6 (1.5)	1.8 (0.9)	2.3 (1.6)	2.7 (1.3)

Results are the mean percentage (SD) of cells marked with the corresponding monoclonal antibody. * $p < 0.05$ compared with the other groups.

intrinsic factor administration; and (d) satisfactory response to parenteral vitamin B-12.

Seventeen healthy individuals, 10 women and seven men, mean age 63.5 years were used as controls.

CULTURE MEDIUM AND REAGENTS

RPMI-1640 (Microbiological Associates, Walkersville, MD) supplemented with 1% L-glutamine (Flow Lab, Irvine, UK), 0.5% HEPES (Flow Lab), and 1% penicillin-streptomycin (Difco Lab, Detroit, MI) was used for cultures. This will be referred to as complete medium. Monoclonal antibodies CD2 (OKT 11), CD3 (OKT 3), CD4 (OKT 4), CD8 (OKT 8) were purchased from Ortho-mune, NJ, USA; CD25 (anti-TAC) from Coulter Clone, FL, USA; and CD16 (Leu 11b) from Becton Dickinson, CA, USA. Recombinant interleukin 2 (rIL2) was provided by Hoffman-La Roche, NJ, USA.

PROLIFERATION STUDIES AND IL2 PRODUCTION

Peripheral blood mononuclear cells (PBMC) were obtained from the heparinised venous

Figure 1: Proliferative response of phytohaemagglutinin (PHA)-stimulated T cells after the addition of recombinant interleukin 2 (rIL2). Results are expressed as mean (SD) counts per minute (cpm). The defective response of T cells from the patients in the presence of PHA was not corrected by the addition of rIL2.

blood of the subjects by Ficoll-Hypaque gradient centrifugation (Nygard Co, Oslo, Norway). T cells were purified by rosetting with sheep red blood cells as previously described.¹⁰ Monocyte contamination was always below 5%. T lymphocytes (50 000 cells/well) were cultured on 96 flat bottom culture plates. Soluble phytohaemagglutinin (PHA; 10 µg/ml; Difco Lab, Detroit, MI, USA), phorbol 12-myristate 13-acetate (PMA; 10 ng/ml; Sigma, St Louis, MO, USA), and rIL2 (10 or 100 U/ml) were added at the beginning of proliferation studies. Each reagent was tested in dose-response titrations before use. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for five days. Eighteen hours before the end of incubation, 1 µCi of ³H-thymidine (Radiochemical Centre, Amersham, UK) was added. The cells were harvested and results expressed as mean counts per minute (cpm) of triplicate cultures.

Lymphokine enriched supernatants were obtained by culturing T cells at 37°C at a density of 5 million cells/ml in complete medium with 10% fetal calf serum (FCS). Cultures were incubated in the presence or absence of PHA (10 µg/ml) and supernatants were harvested at 24 and 72 hours of incubation and stored at -20°C until use. IL2 activity was determined in the supernatants of T cell suspensions by evaluating the dose dependent proliferation induced in the cytotoxic murine line CTL-L2 by the lymphokine present in the cultures.

CELL SURFACE IMMUNOFLUORESCENCE

For indirect immunofluorescence, 0.5 million PBMC were incubated with saturating amounts of the indicated antibodies for 30 minutes and washed three times in phosphate buffered saline supplemented with 1% FCS. Afterwards, they were incubated with fluorescein-conjugated F(ab)'₂ goat anti-mouse immunoglobulin antisera (Kallestad, Austin, TX) for an additional 30 minutes, and then washed three times. The entire procedure was performed at 4°C. The surface immunofluorescence was analysed by means of an EPICS-S flow cytometer equipped with a 2 W argon laser set at the 488 nm line.¹¹

STATISTICAL METHODS

The data from the groups were compared using the Mann-Whitney U test for unpaired samples. A p value of less than 0.05 was considered significant.

Results

PHENOTYPIC STUDY OF PURIFIED T CELLS

Statistical analysis showed no significant differences in the percentage of cells marked with monoclonal antibodies anti-CD2, anti-CD4, anti-CD8, and anti-CD25 among the different groups of patients and controls. The CD4/CD8 ratio was similar for the different groups. CD3+ cells were significantly

decreased and CD16+ cells were significantly increased in the group without pernicious anaemia compared with the other groups and the healthy controls (Table I).

BLASTOGENIC RESPONSE OF PURIFIED T CELLS TO POLYCLONAL MITOGENS AND IL2 SECRETION QUANTIFICATION

The baseline proliferative response of T cells (cells and culture medium only) was low and similar in the three patient groups and the controls. DNA synthesis after five days in the presence of PHA was significantly lower in the patients than the controls; there were no significant intergroup differences. The blastogenic response to PMA was similar in patients and controls (Table II).

Addition to the culture medium of exogenous rIL2 resulted in a slight increase in the T cell blastogenic response. The defective response of T cells from the patients in the presence of PHA was not corrected by the addition of rIL2 to saturating concentration, and there was no significant increase in the mitogenic response (Fig 1).

There were no statistical differences in the low rates of spontaneous production of IL2 by T cells from patients and controls. Likewise, similar levels of IL2 production were found in

PHA stimulated T cells from the three groups of patients and healthy controls (Table III).

PROSPECTIVE STUDY OF FIVE PATIENTS WITH PERNICIOUS ANAEMIA BEFORE AND AFTER TREATMENT WITH VITAMIN B-12

Phenotypic study of purified T cells

There was no difference in the percentage of cells marked with the anti-CD2 MAAb. At six months, however, there was a decrease in the anti-CD3 cells, and an increase in the CD2+ CD3- population (NK cells). The percentages of cells marked with the anti-CD25, anti-CD4 and anti-CD8 Mabs, and the CD4/CD8 ratio were similar at baseline, two and six months (Table IV).

Proliferative response of purified T cells stimulated with PHA and rIL2

Purified T cell proliferation in response to PHA was lower at baseline and after six months of treatment than at two months (Fig 2). There was no significant modification of the proliferative response after supplementation of the culture medium with PHA+rIL2 (Fig 2).

Discussion

The study of the pathogenesis of pernicious anaemia has been hampered by the existence of a simple and effective treatment, namely intramuscular injection of hydroxycobalamin. Although the antibody response has been extensively investigated, little is known to date about the cellular immune response in peripheral blood.

Our phenotypic analysis did not show changes in the CD4/CD8 ratio in any group of patients. Previous studies show contradictory results with regard to the CD4/CD8 ratio. Imamura *et al*¹² described three untreated patients with pernicious anaemia in whom they found an increase in the CD4/CD8 ratio secondary to a decrease in CD8+ T lymphocytes. Wodzinski *et al*¹³ studied 23 patients with pernicious anaemia and found an increase in the CD4/CD8 ratio in those with anti-intrinsic factor antibodies. Soler *et al*¹⁴ found no phenotypic alteration in six patients with untreated pernicious anaemia. Carmel *et al*¹⁵ found no phenotypic differences in their 40 patients with pernicious anaemia (34 treated, six untreated). We have found an increase in the CD16+ cells and a decrease in the CD3+ cells in patients with autoimmune gastritis but without pernicious anaemia. The follow up study of five patients with pernicious anaemia showed a significant increase in the CD2+ CD3- lymphocyte population after six months of treatment. This phenotype corresponds to the NK cells, but the low number of patients makes the significance of this finding uncertain.

There are few studies of the function of peripheral blood lymphocytes in autoimmune gastritis. MacCuish *et al*⁷ showed a hypoprolif-

TABLE II T cell proliferative response at baseline and after addition of phytohaemagglutinin (PHA) and phorbol myristate acetate (PMA), in patients with autoimmune gastritis only, untreated pernicious anaemia, and treated pernicious anaemia and the control group

Mitogen	Chronic autoimmune gastritis	Pernicious anaemia	Treated pernicious anaemia	Controls
Baseline	312 (221)	460 (433)	489 (542)	371 (439)
PHA	69 727 (44 288)*	66 752 (26 657)*	69 072 (23 134)*	122 227 (43 165)
PMA	30 121 (12 433)	37 499 (11 137)	35 135 (10 852)	40 107 (13 396)

Results are expressed as the mean cpm (SD). *p<0.05 compared with the control group.

TABLE III Interleukin 2 activity in the supernatant of phytohaemagglutinin stimulated T cell cultures at 24 and 72 hours

Time	Supernatant dilution (%)	Chronic autoimmune gastritis	Pernicious anaemia	Treated pernicious anaemia	Controls
24 h	25	15 758 (9338)	16 731 (4474)	16 814 (4722)	15 869 (3125)
	12	13 591 (9350)	11 591 (4941)	12 024 (5119)	11 278 (4032)
	6	7 970 (6433)	6 541 (3484)	7 223 (4375)	7 462 (3015)
72 h	25	11 543 (6312)	10 015 (3880)	10 542 (4353)	10 259 (3803)
	12	8 015 (5412)	6 893 (2892)	7 032 (3532)	7 596 (2649)
	6	5 024 (3516)	5 125 (2891)	6 519 (3002)	6 817 (2111)

Results are expressed as mean in cpm (SD). Differences are not statistically significant.

TABLE IV Phenotypic study of purified T cells from five patients with pernicious anaemia (PA) before (baseline) and after two and six months of replacement therapy with parenteral vitamin B-12

Phenotype	Patients with PA			Controls
	Baseline	After 2 mth treatment	After 6 mth treatment	
CD2+	88.6 (5.9)	85.0 (4.6)	83.0 (8.8)	89.6 (2.0)
CD3+	75.4 (5.0)	75.0 (7.2)	64.0 (10.6)*	78.4 (3.1)
CD2+CD3-	12.4 (9.7)	10.0 (4.3)	19.0 (7.2)*	11.2 (2.2)
CD4+	39.2 (3.8)	50.8 (13.3)	44.2 (10.2)	42.2 (6.8)
CD8+	28.8 (12.4)	30.4 (14.3)	22.4 (5.7)	39.2 (6.6)
CD4/CD8	1.7 (1.0)	2.4 (2.3)	2.0 (0.5)	1.2 (0.4)
CD25	3.2 (5.2)	2.6 (1.0)	2.5 (2.3)	3.1 (1.6)

Results are the mean percentage (SD) of cells marked with the corresponding monoclonal antibody. *p<0.05 compared with the other groups.

Figure 2: Blastogenic response of purified T lymphocytes to phytohaemagglutinin (PHA) with or without recombinant interleukin 2 (rIL2) in patients with pernicious anaemia before treatment and after two and six months of treatment with parenteral vitamin B-12. Results are expressed as mean (SD) counts per minute (cpm). The blastogenic response increased significantly after two months of treatment (* $p < 0.05$ with respect to patients untreated and treated for six months), reaching levels found in normal controls. After six months, however, the response was no different from pretreatment. There was no significant modification of the proliferative response after rIL2 addition.

erative response to PHA in the PBMC of 20 patients with pernicious anaemia treated with vitamin B-12. They considered the hypoproliferation secondary to a failure of the intranuclear incorporation of thymidine, and not to an intrinsic defect of blastogenesis. Kätäkä,⁸ however, found no significant differences in the PHA induced proliferation of PBMC of patients and controls, either before or after vitamin B-12 treatment. These studies, however, used PBMC instead of the purified T cells we have analysed. This methodological aspect is relevant since the numbers of T lymphocytes can differ, and their functional state can be modified by the considerable number of non-T cells present in the sample.

We began the functional study of T cells by measuring their proliferative capacity when induced by phytohaemagglutinin. As PHA is a lectin that interacts with multiple surface molecules, we investigated the blastogenic response to mitogenic molecules that directly interact with protein kinase C (PMA).¹⁶ We cultured 50 000 CD3⁺ T cells for five days. Our current practice is to culture 100 000 cells for three days, but at the time we felt that the other method offered superior discriminating capacity. This could introduce some problems of interpretation, as it is possible that after three days there is some production of endogenous cytokines able to interact with the other populations present and modify the measured effects somewhat. However, as we worked with purified T cells, we do not think this is a likely possibility. It is also worth mentioning that contamination with other cells such as monocytes may also affect the response of the target cell population. However, as mentioned in the

Methods section, the contamination with monocytes was always well below 5%.

All three patient groups had a reduced response to PHA compared with the control group. rIL2 addition did not reverse this anomaly. There were no statistically significant intergroup differences, although there was a trend for the group without pernicious anaemia to show higher responses to PHA than the other two. As the NK cell numbers were higher in this group than in the other patient groups, it is possible that these were normal functioning NK cells, whereas the NK cells in the treated and untreated pernicious anaemia groups may have a defect in their response to IL2. We have no hard data, however, with which to substantiate this. PHA induced IL2 production by T cells and the proliferative response to PMA were similar in patients and controls. These results strongly suggest that T cells from autoimmune gastritis patients have a functional defect that interferes with the generation of the second messengers of the T cell activation process. It is noteworthy that the T cell proliferation deficiency is present in patients with autoimmune gastritis, even when pernicious anaemia is not yet present. Indeed, the intensity of this T cell functional defect remains unmodified in those patients who develop pernicious anaemia, and even in those whose haematological anomalies are corrected by vitamin B-12 treatment. It is remarkable, however, that a transient return to normal of the T cell blastogenic response can be observed early in the treatment with parenteral vitamin B-12. It is possible that this finding is a consequence of the changes induced by vitamin B-12 in nucleic acid metabolic pathways.

In conclusion, we have shown that the three groups of autoimmune gastritis patients studied present a functional defect in the T lymphoid population that is independent of the stage of the disease and of vitamin B-12 treatment. This change might be further characterised using BrdU uptake, Ki-67 expression, and DNA flow cytometric analysis. This T cell immunodeficiency may be part of the immune system dysregulation that is presumably involved in the pathogenesis of the gastric lesions of these patients.

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