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CD69 expression on α-gliadin-specific T cells in coeliac disease

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SUMMARY

Coeliac disease (CD) is a T-cell mediated immunological disease of the small intestine which is triggered in susceptible individuals by ingestion of gluten. The pathogenic mechanism of coeliac disease, and the role that α-gliadin specific T cells play in mucosal lesions and their involvement in peripheral blood is not yet explained at all. Previous studies have reported proliferative response to α-gliadin measured with the classic assay of ^{3}HTdR incorporation. We analysed the activation antigen CD69 on T cells from CD patients and normal individuals following stimulation with α-gliadin and different antigens (tetanus toxoid, peptides unrelated to gliadin and PHA). CD69 coexpression with T cell CD3+ and proliferation marker Ki67 was evaluated with time.

CD69 coexpression with T cell CD3+, CD4+ and CD8+ was also evaluated. It was found that peripheral blood mononuclear cells (PBMC) of coeliac patients increased their percentage of CD69 positive T cells when stimulated with α-gliadin, in comparison with cells from controls. Significant T cell activation was found only in subjects not treated with the gluten free diet; a positive response was found also in two coeliac patients with selective IgA deficiency, anti-endomisium negative, without circulating IgA anti α-gliadin or anti-tissue transglutaminase antibodies. The CD69 expression after stimulation was compared with the standard method of ^{3}HTdR incorporation. Our data show that CD69 expression is useful to asses a specific T cell response to α-gliadin in coeliac disease. in a very short time. Moreover, the method allows to investigate T cell response at the lymphocyte subsets level, which represents a useful tool in the diagnosis of coeliac disease.

INTRODUCTION

Coeliac disease (CD) is a malabsorptive disorder characterised by villous atrophy, crypt cell hyperplasia and infiltration of lymphoid cells in small intestine mucosa. Dicke *et al.,* (1953) established that coeliac disease is triggered in susceptible individuals by the ingestion of gluten, the main component of wheat flour. CD is probably an immunological disease and is closely associated with a particular HLA-DQ2 heterodimer (Meuli *et al.,* 1995; Sollid and Thorsby, 1993; Tighe *et al.,* 1993). The pathogenic mechanism which causes mucosal lesions is still unknown, although recently Maki *et al.* (1992) have suggested that CD is an autoimmune disorder and not only an enteropathy; indeed, the disease has

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many features which support the autoimmune hypothesis: production of autoantibodies (antiendomysial antireticulin, antitransglutaminase antibodies), HLA-class II gene susceptibility, presence of immunological changes in patients without clinical signs of disease. However, the role of α-gliadin specific T cells in mucosal damage and their involvement in peripheral blood is still unexplained. Evidence indicates that some CD4+ T cells, using the T-cell receptor located in the lamina propria, may initiate onset of the disease (Halstensen *et al.,* 1993). Previous studies have reported proliferative responses of peripheral blood T cells against gluten proteins both in coeliac patients and in controls with the standard method of ^{3}H-TdR incorporation (Gjertsen *et al.,* 1994). Recently, literature reported that CD69 is the earliest activation antigen expressed on peripheral blood T cells following mitogenic stimulation, while it is undetectable, or detectable at only very low levels, on unstimulated lymphocytes. These characteristics make flow cytometric assessment of CD69 an excellent candidate for the rapid screening of lymphocyte activation *in vitro*. Maino *et al.,* (1995) demonstrated that under certain conditions similar dose response profiles were achieved when the percentage of CD69-expressing lymphocytes at 4 h was compared to the tritiated-thymidine incorporation assay after 72 hours of activation, and that lymphocyte activation to recall antigen can also be predicted by CD69 expression at 4 h following stimulation in whole blood. Observations from other authors (Caruso *et al.,* 1997; Craston *et al.,* 1997; Mardiney *et al.,* 1996; Simms *et al.,* 1996) suggest that CD69 may constitute a rapid and sensitive marker for quantifying T-cell subset responses to a variety of different stimuli: PHA, PMA, alloantigen, cytokine, but there is not yet evidence of application in food antigen. Here we report studies on the expression of CD69 on T cells from peripheral blood of coeliac patients and a group of healthy controls stimulated with α-gliadin, tetanus toxoid, PHA, and with a mixture of two peptides unrelated to α-gliadin. Comparing the results with the classical method of incorporation of ^{3}H-TdR, we demonstrated that this technique is rapid and sensitive, avoiding radioactive waste and allowing to assess immunological involvement of T cell subsets in peripheral blood of coeliac disease, which is an additional diagnostic tool in cases of IgA deficiency.

MATERIALS AND METHODS

Cell donors
The coeliac patients (CD) studied (n=36, age 0-43) were diagnosed according to European Society for Pediatric Gastroenterology and Nutrition criteria of 1970. Twenty one patients were untreated (UCD), 19 of which were positive in tests for anti α-gliadin antibodies (AGA), anti-tissue transglutaminase (tTg) and anti-endomysium antibodies (EMA) which are markers for coeliac disease; only two were negative, but resulted to be suffering from selective IgA deficiency with a borderline value for AGA and tTg IgG . Fifteen patients were treated with a gluten free diet (CD-GFD) and had normal antibody levels. As controls were tested 7 subjects with other intestinal diseases (2 with Crohn's disease, 3 with inflammatory bowel disease, 2 with chronic diarrhoea) and 16 normal volunteers aged 24 to 45, recruited from the hospital staff. Peripheral blood was collected by venipuncture with sodium heparin as anticoagulant.

Cell culture
Peripheral blood mononuclear cells (PBMC) were purified by density gradient centrifugation with Lymphoprep (Nycomed Pharma, Norway), washed in Hank's balanced salt solution, and resuspended in RPMI 1640 media (Seromed SPA, Milan, Italy) supplemented with 10% FCS serum (Seromed), 2 mM glutamine and antibiotics. Cell density was adjusted to 10^6 cells/ml. 1 x 10^6 cell samples were aliquoted in duplicate into sterile polystyrene round bottom tubes with caps (Becton Dickinson, Milan, Italy) at a final volume of 500 µl, in medium alone (unstimulated control) or with the following antigens: α-gliadin (200 µg/ml), tetanus toxoid (0.5 U/ml) (Chiron S.p.A., Siena, Italy), a mixture of two peptides unrelated to α-gliadin (200 µg/ml), PHA (1 µg/ml) from Seromed. Cells were cultured in a humidified incubator at 37°C in 5% CO_2, samples were harvested for phenotyping and analysis at 4-12-24-48 h, an aliquot of cells was counted in trypan blue for an estimation of viable cells (over 95% viability).

Antigens
α-gliadin was prepared from wheat flour according the method of Bernardin *et al.,* (1967).
The two peptides unrelated to α-gliadin (the fragment 11-25 of Bac5 and the sequence GARSER-

FRP) were prepared as previously described (Frank *et al.,* 1990). They were synthesized by the solid phase method on a Milligen 9050 synthesizer using Fmoc chemistry. The syntheses were performed with Pro-substituted PepSyn-KA resin (0.1 mmol/g) using a five-fold excess of amino acid, and coupling reagents (N-hydroxy-benzotriazole and 2-(1H-benzotriazole-1-y1)-1,1,3,3-tetramethy-luronium tetrafluoroborate). After deprotection and cleavage, the peptides were purified by RP-HPLC using a 0-60% water/acetonitrile gradient in 0.1% trifluoroacetic acid, and their molecular mass was determined with an API I ion spry mass spectrometer (PE SCIEX, Toronto, Canada).

^{3}H-TdR Assay

PBMC were isolated as described, $1x10^5$ PBMC were added to appropriate wells of a 96-well U bottom microtiter plate (Costar, Milan, Italy), in 200 µl of culture medium alone or containing mitogens or antigens. The medium was composed of RPMI 1640 supplemented with 10% heat-inactivated calf fetal serum, 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomicin. Cultures were incubated at 37°C for 72 h (PHA) or 120 h (tetanus toxoid, α-gliadin and unrelated peptides) under the same experimental conditions used for culture for flow cytometry. 16-18 hours before harvesting, cells were pulsed with 1 µCi ^{3}H-TdR (Amersham, UK) in 20 µl of medium and cultured. Cells were then harvested onto glass fiber filters with a Cell Harvester (Skatron Instruments, Finland) for determination of ^{3}H-TdR incorporation by scintillation counting. Lymphocyte proliferative responses were reported in the form of the average ^{3}H-TdR incorporation of replicate stimulated lymphocyte cultures minus the average of the blank.

Immunophenotyping and fluorescence staining for CD69 and Ki67 expression

Stimulated or unstimulated PBMC were analysed by three-or two-colour flow cytometry. Before labelling, cell cultures were washed twice in HBSS 1.0% BSA and aliquoted in 50 µl for each test tube. The following MoAbs (Becton Dickinson) were used: CD3 PerCP, CD69 PE, CD4 or CD8 FITC. Labelling was performed for 30 min at 4°C followed by a wash step. Samples of whole blood were lysed with Facs lysing solution (Becton Dickinson). In order to evaluate correlation between a proliferation index and CD69 expression, a selected number of samples were stained with CD3PerCP, CD69PE and Ki67 FITC (DAKO S.p.A., Milan, Italy) according to the method proposed by the manufacturer (Schwarting *et al.,* 1986) with minor modifications. Briefly, the pellet of $1x10^6$ cells stained for surface antigens was fixed with 100 µl of solution A of FIX and PERM kit (CALTAG) for 15 min at RT, washed twice and resuspended in 100 µl of solution B with 10 µl of Ki67 FITC. IgG isotype control antibody conjugates (Becton-Dickinson) were included to establish background fluorescence. All samples were then washed and resuspended in 0.5 ml of PBS for flow cytometric analysis.

Flow Cytometry

Live gating was used to collect 10,000 events within the T-cell gate defined by SSC and FL3 for CD3+ cells. Analysis was performed using Consort 32 and LYSYS II software. The percentage of CD69+cells within CD3, CD4 and CD8 were determined both for unstimulated and antigen-stimulated samples on the basis of two-colour dot plots (FL1 vs FL2). The percentage of Ki67 positive cells was determined among the CD3+ gated cells. For all experimental conditions, matched subclass controls were employed to determine the level of non-specific binding. Data were expressed as the percentage of CD69 positive cells subtracting the value of unstimulated samples from the stimulated.

Method for determination of circulating antibodies markers of celiac disease

Circulating AGA and tTg antibodies of IgA and IgG class were measured by an ELISA method developed in our laboratory (Perticarari *et al.,* 1992). In brief, 100 µl of 1:200 serum sample dilutions in PBS, were placed in wells of a microtiter plate pre-coated with 2 µg/well of α-gliadin or 1 µg/well of guinea pig tissue transglutaminase (SIGMA). Binding of antibodies was revealed with goat anti-human IgA or IgG phosphatase-conjugated, followed by incubation with a phosphatase substrate solution (PNPP 1 mg/ml in carbonate buffer). Colour development was read at 405 nm with a Vmax ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA). All tests were performed in duplicate and results were expressed as percent of optical density of a positive control constituted of a pool of ten sera from coeliac patients previously

measured and resulted positive. IgA anti-endomysial antibodies were performed on cryostatic sections of umbilical cord vein by indirect immunofluorescence, and evaluated by microscopy

Statistical analysis

One-way ANOVA was performed using Graph Pad Prism version 3.00 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com.

RESULTS

CD69 expression after stimulation with α-gliadin, PHA and other antigens

CD69 expression started after 4h of α-gliadin stimulation (200 µg/ml final concentration) in coeliac samples, a peak was reached between 24 h and 48 h. Conversely, CD69 expression in unstimulated controls was baseline and remained almost constant up to 48 hours. A representative example of double staining for CD69 expression and for proliferation marker Ki67, after stimulation of PBMC from a coeliac patient, are shown in Fig. 1, with 200 µg/ml α-gliadin (upper plots) and 1 µg/ml PHA (low plots). Plots depict response of stimulated samples for 4, 24 and 48 hours, respectively. An increase of CD69 was observed both with α–gliadin and with PHA yet even 4 hours, while Ki67 rose to detectable values only after 24 hours (8.6% with α–gliadin and 31.4% with PHA). After 48 hours, the percent of cells double stained for CD69 and Ki67 rose to 14.5% for α-gliadin and 46% for PHA. In this analysis, the simultaneous expression of CD69 and Ki67 was determined on the CD3+ gated cells.

We evaluated the CD69 expression on T lymphocyte subsets CD3, CD4, and CD8, after α-gliadin stimulus in 21 coeliac patients and in 16 healthy controls; results are calculated by subtracting value of unstimulated sample from the stimulated, and are presented in Table I. All three subsets responded almost identically to α-gliadin stimulus in the coeliac patients in comparison with the healthy controls, but the most statistically significant difference between the two populations was due to the CD69+CD4+ cells (p <0.0001). On the basis of these data, we chose the CD69 expression within the CD4 subset to discriminate between positive and negative populations.

Coeliac patients responded similarly to controls to tetanus toxoid and PHA (Table II) but did not respond to a mixture of peptides unrelated to α-gliadin, indicating a specific response to α-gliadin in CD. Representative contour plots of CD69 expression within CD4 in response to α-gliadin and unrelated peptides from a coeliac individual (A,B,C) and from a healthy control (D,E,F) are presented in Fig. 2.

CD69 expression after 24 hours of culture was comparable with results obtained with the standard method of ^{3}H-TdR incorporation after 72 hours of stimulation with PHA and 120 hours with antigens (Table II). No changes in the proportion of lymphocyte subsets, in viability or in leukocyte counts determined before culture and over the time course were observed (data not shown).

Table I

CD69 expression on T cell subsets after *in vitro* stimulation with α-gliadin, 200 µg /ml

	CD3+	CD4+	CD8+
Untreated Coeliac Disease (n=21)	19.24 ± 10.61	12.76 ± 9.36	6.24 ± 3.07
Healthy Controls (n=16)	9.24 ± 5.07	1.39 ± 0.96	4.09 ± 3.07
p value	<0.0007	<0.0001	<0.0100

Values are means ± standard deviation (SD) calculated as the net percent of activated cells subtracting the percent of unstimulated samples. Cells were collected and stained after 24 hours of incubation.
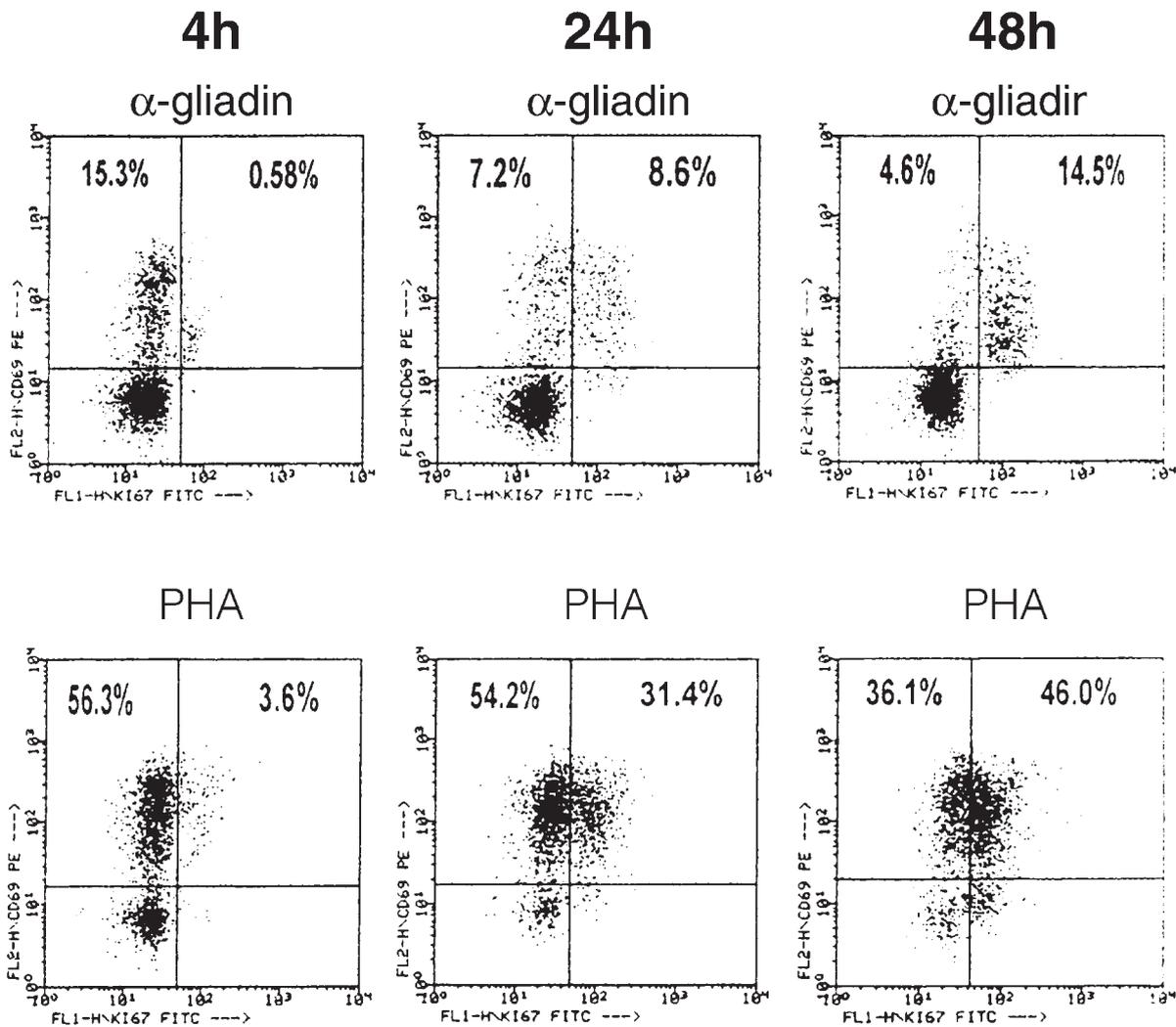
Fig. 1 - Example of double staining for CD69 expression and Ki67 proliferation marker of lymphocytes from a coeliac patient stimulated with α-gliadin (upper plots) and PHA (low plots). Cells were stimulated for 4, 24 and 48 hours, and then stained with CD69-PE, Ki67-FITC and CD3-PerCP. An electronic gate was set on CD3+ lymphocytes and FL1, FL2 were measured for Ki67-FITC and CD69-PE (percentages of double staining are indicated in the respective quadrants).

Relationship between CD69 activation after a-gliadin stimulation and coeliac disease

The mean percentage value of CD4+ cells expressing CD69 following α-gliadin stimulation in untreated coeliac disease (mean 12.76 ± 9.36) was statistically different ($p<0.0001$) from that observed in healthy individuals (mean 1.3 ± 0.9), from that in coeliac subjects on a gluten-free diet ($2,8 \pm 3.5$), and from that in a group of controls with other intestinal diseases (mean 2.25 ± 2.31) (Fig. 3, and Table I).

None of the lymphocyte samples obtained from healthy individuals or from controls with other intestinal diseases showed a significant increase in activation marker expression on CD4+ cells after 48 h of *in vitro* stimulation with α-gliadin (200 µg/ml). Conversely, 3 of the 15 coeliac patients on gluten-free diet had a significant response to recall antigen α-gliadin. To discriminate positive from negative populations, we established a cut-off as mean value plus three standard deviations of percentage of CD4+CD69+ determined in 16 healthy controls, as
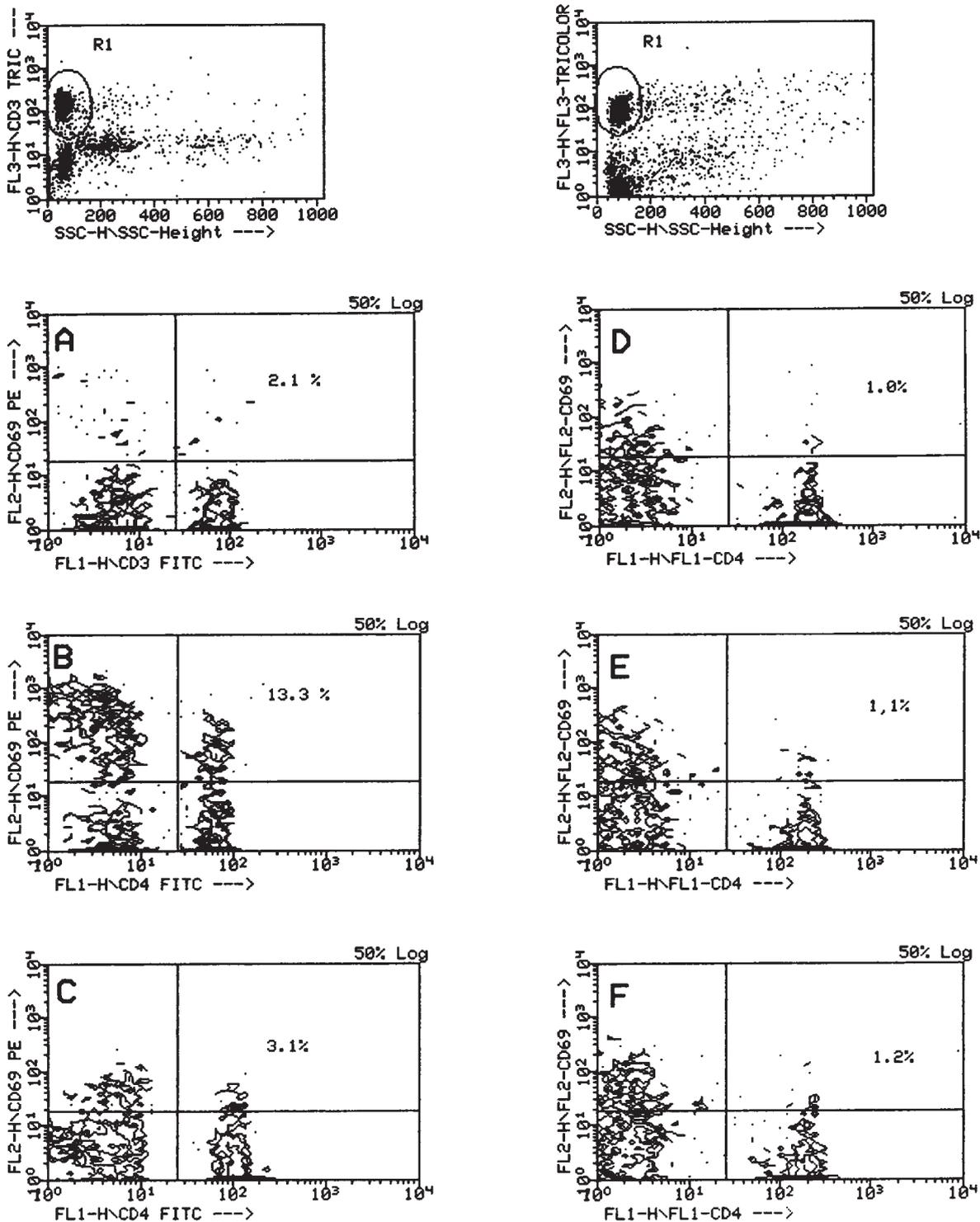
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Fig. 2 - Three-colour immunofluorescence analysis showing CD69 expression on CD4+ activated lymphocytes. Representative plots from a coeliac patient (left), and from a healthy control (right) are shown. Samples were cultured for 24 hours with medium alone (A-D), with 200 µg/ml α- gliadin (B-E), with 200 µg/ml of a mixture of two peptides unrelated to α-gliadin (C-F). A gate R1 was established on the CD3 positive lymphocytes versus SSC. The percentage of double stained cells is indicated in the upper right quadrant.

Table II
Comparison between T cell-CD69 expression and ^{3}H-TdR incorporation at 120 hours in coeliac patients
and healthy controls after PBMC stimulation

		α-Gliadin (200 µg/ml)	unrelated peptides (200 µg/ml)	Tetanus Toxoid (0.5 U/ml)	PHA (1 µg/ml)
Untreated Celiac	CD3+CD69+ [a]	19.24 ± 10.61	2.6 ± 1.32	18.2 ± 4.91	40.12 ± 10.97
Disease (n=21)	cpm x 10^3 [b]	20.7 ± 5.24	1.54 ± 3.31	13.3 ± 6.55	54.81 ± 9.32
Healthy Controls	CD3+CD69+[a]	9.24 ± 5.07	2.4 ±1.17	23.06 ± 9.21	41.39 ± 12.63
(n=16)	cpm x 10^3 [b]	5.1 ± 1.42	2.6 ± 0.92	17.9 ± 12.63	57.43 ± 10.25

[a] time = 24 h, percentage of cells expressig CD69.
[b] time =120 h, for antigens or 72 h for PHA.

shown in Fig. 3. Table II illustrates results obtained stimulating PBMC from 21 coeliac patients with a mixture of two peptides unrelated to α-gliadin, and with tetanus toxoid as a control of specificity; no activation was demonstrable in healthy controls (mean % CD69=1.39). None of patients or controls responded to the unrelated peptides, whereas coeliac patients, when immunized, responded to tetanus toxoid as well as other individuals.

These results suggest that coeliac patients respond specifically to α-gliadin; moreover, we found that two subjects negative for EMA and without AGA and anti tTg IgA circulating antibodies, had a specific T-cell activation. The two subjects that presented

also an IgA deficiency were then confirmed as coeliac patients by jejunal biopsy.

DISCUSSION

CD69 is a lymphoid activation antigen whose rapid expression makes it suitable for the detection of T-cell activation and for subset activation analyses. Many authors have reported the utility of flow cytometric detection of T-cells activated with antigenic or mitogenic stimuli (Caruso et al., 1997; Craston et al., 1997; Maino et al., 1995; Mardiney et al., 1996; Simms et al., 1996). A
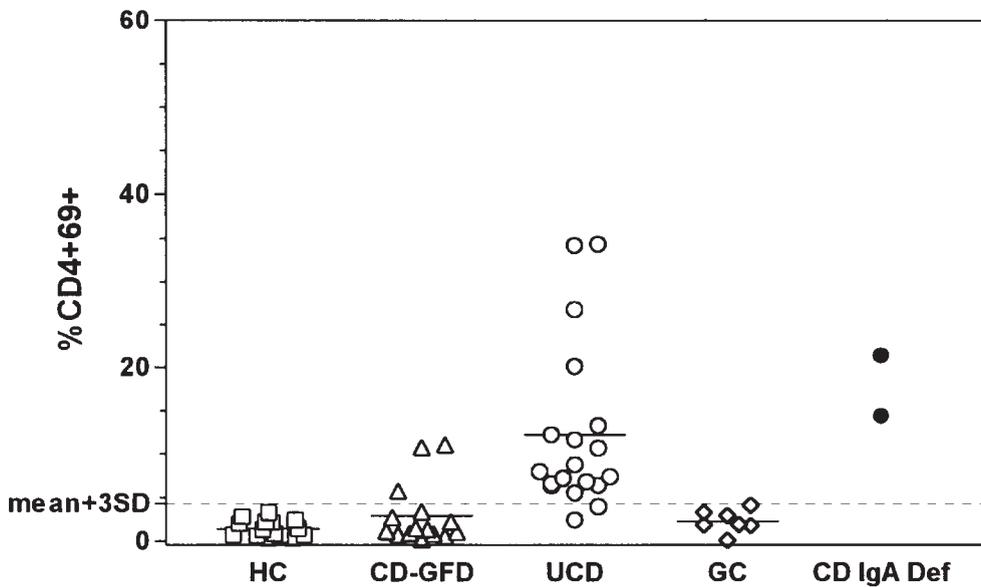

Fig. 3 - CD69 expression on CD4+ cells of α-gliadin stimulated lymphocytes from each group studied: healthy controls HC (squares), coeliac disease on gluten free diet CD-GFD (triangles), untreated coeliac disease UCD (circles), gastroenterological controls GC (rhombs) and CD with IgA deficiency CD-IgA def (filled circles). The horizontal line indicates the mean +3 SD of values from healthy controls that was assumed as the cutoff point discriminating positive from negative.

method which might replace standard tritiated thymidine incorporation. Our findings are similar to those of other authors, indicating that this method allows the evaluation of mitogenic response to PHA, and also responses to food antigens and non food antigens in PBMCs in a very short time. Since CD69 is a marker of early activation, its expression may not necessarily reflect late activation events or correlate with proliferation, as demonstrated in an experiment with simultaneous staining with Ki67. As shown in Fig. 1, the increase in CD3+CD69+ cells after stimulus with α-gliadin in the peripheral blood of a coeliac patient, precedes the expression of Ki67; after 4 hours of incubation the proliferation marker Ki67 is not yet detectable, while the CD3+CD69+ lymphocytes reached 15.3%; after 48 hours of incubation, the CD3+CD69+ lymphocytes had risen to 19.1%, of which 14.5% simultaneously expressed Ki67. These data are in agreement with Lopez *et al.,* (1991). We demonstrated that 24 hours of stimulation with α–gliadin was sufficient to discriminate between responses of coeliac patients and controls; this makes the method rapid and suitable for laboratory practice.

We applied this CD69 flow cytometric method to determine if the response to α-gliadin stimulation in CD is an aspect of the specific T cell-mediated hypersensitivity to gluten, and particularly to the gluten fraction, α-gliadin. This aspect is reported to be important in the immunopathogenesis of CD, as demonstrated by other authors by the finding of an increased number of CD4+CD25+ activated T cells in the lamina propria of untreated coeliac patients. This overstimulation can be reproduced by cultivation of biopsy specimens from coeliac patients with gluten peptides *in vitro* (Sturgess *et al.,* 1994) and was also demonstrated in peripheral blood of coeliac patients by a proliferative T cell response to gliadin as measured by the ^{3}H-thymidine incorporation assay (Gjertsen *et al.,* 1994). We also obtained a confirmation of a proliferative response to α-gliadin in coeliac patients by ^{3}H-TdR incorporation, but this method does not permit elucidation of T lymphocytes subsets. Our data show that all T lymphocytes, but particularly CD4+ T cells of untreated coeliac patients, were responsive to α-gliadin and increased their expression of CD69, while coeliac patients on gluten-free diet, and healthy or other gastroenterological controls did not respond significantly to α-gliadin stimulus. We also found a mild activation response to α-gliadin stimulation in two coeliac patients on a gluten-free diet in whom humoral markers of the disease were normal. This finding could be explained by compliance or duration of the diet which leads antibody levels to normalize yet allows memory cells, able to respond to α-gliadin stimulus, to remain.

Jensen *et al.,* (1995) demonstrated a proliferative α-gliadin specific T cell response also in healthy individuals without correspondingly increased levels of anti α-gliadin, and reported that only individuals carrying DQ2 with α-gliadin-specific T cells in their peripheral blood also populated their intestinal mucosa. This report would appear to contradict our data. However, recent studies have introduced the concept of silent, latent and potential coeliac disease; in these forms the disease can go undiagnosed for years. Silent CD is marked by mucosal damage without clinical symptoms, both latent and potential CD are characterized by almost normal jejunal mucosa. Latent coeliac patients are often asymptomatic although endomysial antibodies are present as a predictor of the disease. The term "potential CD" has been proposed for those subjects who do not have consistent alterations in a jejunal biopsy but have immunological abnormalities similar to those found in coeliac patients (Troncone *et al.,* 1996). Sollid and Scott (1998) reported that patients with coeliac disease with clinical manifestations represents only 30%-40% of the entire spectrum of gluten sensitised individuals. Because of lifelong disease and a possible predisposition to cancer, the earliest possible diagnosis is important and patients should be advised to adhere to a strict gluten free diet for life. Serum circulating antibodies to gluten, tissue transglutaminase and endomysium are currently used in screening for coeliac disease, but any one test alone is not yet suitable as the gold standard for coeliac disease large-scale screening. Recent studies (Dieterich W. *et al.,* 1998; Lock R.J. *et al.,* 1999) have reached the conclusion that IgA anti tTg is a well suited tool to detect untreated coeliac disease. Nevertheless Sulkanen *et al.,* (1998) show that the titers of IgA autoantibodies to tTg fluctuate with dietary gluten exposure, whereas gluten reactive CD4+T cells persist in the small intestinal mucosa of coeliac patients.

Because of the association of selective IgA deficiency with coeliac disease as reported by Collin *et al.,* (1992), Cataldo *et al.,* (1997), patients should be assessed for IgG class, but all tests for IgG have poor disease specificity, i.e. it is well known that AGA-IgG and tTg-IgG occur also in other gastrenterological diseases. The method that we propose represents a useful tool to confirm the diagnosis of gluten-sensitive enteropathy, even in subjects with IgA deficiency, in peripheral blood, thus avoiding biopsy.

In addition, we demonstrated that the method might replace standard tritiated thymidine incorporation as a rapid and reproducible measurement of lymphocyte activation even by alimentary antigens, thus avoiding radioactive waste.

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