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Association of increased numbers of peripheral blood double-negative T-lymphocytes with elevated serum IgG levels in severely handicapped children

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Abstract CD3⁺4⁺8[−] double negative cells in peripheral blood lymphocytes were examined in 21 severely handicapped children divided into two groups according to serum IgG level. All children were bedridden and were taking multiple anticonvulsants and there were no apparent clinical differences between these two groups. Serum levels of IgG correlated well with percentages of CD3⁺4⁺8[−] double negative lymphocytes in patients of both groups. In comparisons between the two groups, the high IgG group had higher counts of CD3⁺4⁺8[−] double negative lymphocytes in peripheral blood than the normal IgG group. Two distinct types of double negative cells were identified in the patients with high IgG: one had T-cell antigen receptors of $\gamma\delta$ heterodimers, the other had receptors of $\alpha\beta$ chains on their surface. As double negative T-cells are reported to have an important role in defence against bacterial infections, the increased numbers

of CD3⁺4⁺8[−] T-cells of both phenotypes in the high IgG patients may reflect exposure to repetitive bacterial stimuli or persistent subclinical infection which in many cases, may be undetectable clinically. Moreover, the hyperimmune states shown by the high serum IgG of these patients may result from the appearance of these unique lymphocytes because they are reported to have a helper function for IgG synthesis *in vitro*. Taken together, the increased numbers of double negative cells in patients with high IgG may reflect activated defence mechanisms and the development of hyperimmune status.

Key words Double negative T-lymphocytes · T-cell receptors
Severely handicapped · IgG level
Infectious diseases

Abbreviations TCR T-cell receptors
FITC fluorescein isothiocyanate
PE phycoerythrin

Introduction

Severely handicapped children experience repeated infections which tend to be more difficult to treat than those experienced by normal healthy individuals. Elevated serum immunoglobulin levels in severely handicapped individuals may be the result of chronic subclinical inflammation [28]. Analyses of peripheral lymphocytes that would explain the cause of the increase of serum Ig in the

sera of such patients have yet to be done. Existing reports on the abnormalities of lymphocyte subpopulations [37], impaired lymphocyte responses to mitogens [18, 44], and decreased delayed type cutaneous hypersensitivity [18] are not directly related to the overproduction of IgG.

The role of T-cell antigen receptors (TCR) in antigen recognition has been clarified and two types of TCR have been identified [7]. Conventional heterodimeric $\alpha\beta$ -TCR are expressed by the majority of peripheral blood T-cells, most of which also co-express CD4 or CD8 as accessory

molecules for antigen recognition. Minor populations of TCR- $\alpha\beta^+$ cells, however, which bear neither CD4 nor CD8 [29] are called double negative T-cells. Another receptor type is TCR- $\gamma\delta$, and most T-cells that bear it are double negative [7].

Functional analyses have shown that both types of double negative cells retain potent cytotoxic activity which includes non-specific [6, 8, 23, 32] and major histocompatibility linked cytotoxicities [10, 21, 36]. Moreover, Strober et al. [43] have described natural suppressor clones of TCR- $\alpha\beta^+$ CD3 $^+$ 4 $^-$ 8 $^-$ which show non-major histocompatibility restricted inhibition of mixed lymphocyte cultures.

In addition to their cytotoxicities, TCR- $\gamma\delta^+$ T-cells respond vigorously to bacterial stimuli [1, 27], an indication that TCR- $\gamma\beta^+$ T-cells function as one of the initial effectors against invading micro-organisms. Similarly, double negative TCR- $\gamma\beta^+$ cells in mouse liver [2] and human T-cell clones of the same receptor type [33] respond to bacteria. Besides proliferative responses, double negative TCR- $\gamma\delta^+$ T-cells in vitro augment antibody production both in human [38] and animal models [31].

We investigated the number of T-cell subtypes present in severely handicapped children. Because the presence or absence of infectious disease is difficult to discern in such patients, the evaluation of T-cell profiles might show a part of their immunological backgrounds, allowing us to predict the presence of underlying infectious diseases and to treat patients for such diseases and to achieve profound understanding of the hyperimmune status in these patients.

Patients and methods

Patients

Twenty-one severely handicapped patients in the Moro Hospital were divided into two groups on the basis of serum IgG levels (Table 1). Twelve patients aged 4–16 years had high IgG levels above the mean + 3SD of those of age-matched controls [3]. The remaining nine patients, aged 5–17 years were assigned to the normal IgG group. None of the patients had anti-nuclear antibodies in their sera. All the children were bedridden and were receiving tube feeding because of cerebral palsy, mental retardation, epilepsy, or hydrocephalus. Patients with chromosomal abnormalities, hereditary diseases, progressive central nervous system degeneration, and metabolic disorders were excluded from this study. None of the subjects showed malnutrition, anaemia, or manifestations of active infection when examined. All had been taking multiple anticonvulsants that included phenobarbital, phenytoin, clonazepam, nitrazepam, and valproic acid for more than 4 years. Patients enrolled in this study showed respiratory infections two or three times a year and there were no significant differences between the two groups in clinical status and medication. Within several months of completion of this study, three of the patients in the high IgG group died suddenly: patients 10, 12, and 19 died of intestinal bleeding, hypothermia, and pneumonia, respectively. The study was approved by the Institutional Research and Publications Review Committee of Human Subjects of the Moro Hospital.

Flow cytometric analysis of the T-lymphocyte subset

Mononuclear cells were isolated from heparinised whole blood by Ficoll-Conray density centrifugation [9]. These cells were washed three times with phosphate-buffered saline then resuspended in

Table 1 Clinical data of the patients studied. *M* male, *F*, female, *CP* cerebral palsy, *Epi* epilepsy, *MR* mental retardation, *HC* hydrocephalus

Case	Sex	Age	Diseases	Serum level (mg/dl)		
				IgG	IgA	IgM
<i>Normal IgG group</i>						
1	M	16	CP, Epi, MR	1560	446	348
2	M	8	CP, Epi, MR	956	160	199
3	M	14	CP, Epi MR, HC	1290	189	199
4	M	5	CP, Epi, MR	1410	178	169
5	M	17	CP, Epi, MR	1430	430	219
6	M	12	CP, Epi, MR	1860	438	421
7	F	13	CP, MR	1210	305	170
8	F	10	CP, Epi, MR	1410	112	226
9	F	11	CP, Epi, MR	1600	237	144
<i>High IgG group</i>						
10	M	12	CP, Epi, MR	2100	484	242
11	M	4	CP, Epi, MR	1980	258	316
12	M	6	CP, Epi, MR	1930	258	316
13	M	5	CP, Epi, MR	1950	280	358
14	M	13	CP, Epi, MR	2450	35.6	259
15	M	5	CP, Epi, MR	1510	218	140
16	M	5	CP, Epi, MR	1710	368	599
17	F	16	CP, Epi, MR	2270	157	166
18	F	13	CP, Epi, MR, HC	1990	268	463
19	F	13	CP, Epi, MR, HC	2010	293	421
20	F	16	CP, Epi, MR	2240	384	486
21	F	4	CP, Epi, MR	1880	<29.8	159

Table 2 Surface phenotype of mononuclear cells in peripheral blood from severely handicapped children

	Normal IgG (n = 9)	High IgG (n = 12)
CD3 $^+$	73.9 $\pm$ 7.4	69.8 $\pm$ 10.1
CD4 $^+$	48.1 $\pm$ 12.5	41.6 $\pm$ 14.1
CD8 $^+$	24.8 $\pm$ 8.0	28.2 $\pm$ 9.3
CD4 $^+$ /CD8 $^+$	2.18 $\pm$ 1.13	1.88 $\pm$ 1.41
CD4 $^+$ 45RA $^+$	27.5 $\pm$ 11.5	25.3 $\pm$ 10.0
CD4 $^+$ 45RA $^-$	20.4 $\pm$ 7.5	15.6 $\pm$ 5.8
CD4 $^+$ 45RA $^+$ /CD4 $^+$ 45RA $^-$	1.50 $\pm$ 0.80	1.71 $\pm$ 0.72
CD8 $^+$ 11b $^+$	1.6 $\pm$ 0.8	1.9 $\pm$ 1.1
CD8 $^+$ 11b $^-$	22.8 $\pm$ 8.1	25.9 $\pm$ 9.6
CD3 $^+$ 4 $^-$ 8 $^-$	5.5 $\pm$ 2.1	7.9 $\pm$ 3.2*
CD16 $^+$	11.4 $\pm$ 6.4	11.8 $\pm$ 7.1
CD56 $^+$	7.1 $\pm$ 4.4	5.5 $\pm$ 3.1

Data are the means $\pm$ SD% of positive cells for each group.

* $P < 0.025$

RPMI 1640 medium containing 0.01% sodium azide, after which they were made to react with various combinations of mouse monoclonal antibodies (mAb). The mAb used were fluorescein isothiocyanate (FITC)-conjugated OKT4 (CD4), OKT8 (CD8), TCR-1 (TCR- $\alpha\beta$), TCR δ -1 (TCR- $\gamma\delta$), Leu 11a (CD16), Leu 18 (CD45RA), Mo 1 (CD11b) and phycoerythrin (PE)-conjugated Leu 4 (CD3), Leu 3a (CD4), Leu 2a (CD8), Leu 19 (CD56). OKT4 and OKT8 were purchased from Ortho Pharmaceutical Corp (Raritan, NJ), Leu 4, Leu 3a, Leu 2a, Leu 11a, Leu 18, Leu 19 and TCR-1 from Becton Dickinson (Mountain View, CA), Mo 1 from Coulter Immunology (Hialeah, FL) and TCR δ -1 from T-cell Sciences Inc. (Cambridge, MA). CD3⁺4⁺8⁺ cells were identified by the combination with FITC-conjugated CD4 antibody, FITC-conjugated CD8

antibody, and PE-conjugated CD3 antibody. Similarly, CD4⁺8⁺TCR- $\alpha\beta$ ⁺ cells were identified by combination with PE-conjugated CD4 antibody, PE-conjugated CD8 antibody, and FITC-conjugated TCR- $\alpha\beta$ antibody. Dual colour-stained cells were analysed with a fluorescence activated cell sorter 440 (Becton Dickinson).

Statistical analysis

The two-tailed Student's *t*-test was used to analyse the data obtained for the normal and high IgG groups investigated in this study.

Results

T-cells identified by their surface expression of CD3/TCR were divided into reciprocal subpopulations of CD4⁺ and CD8⁺ T-cells. As shown in Table 2, we found no significant differences between the surface phenotypes of the conventional T-cell markers CD3, CD4, and CD8 of the normal IgG and high IgG groups. CD4⁺ (helper/inducer) cells have been subdivided into CD4⁺45RA⁺ (suppressor inducer) cells and CD4⁺45RA⁻ (helper inducer) cells, the former being reported to be the inducer of CD8⁺ suppressor cells and the latter of helper cells in pokeweed mitogen-driven Ig synthesis [24, 25]. The patients in the high IgG group did not, however, necessarily express the increase in CD4⁺45RA⁻ cells or decrease in CD4⁺45RA⁺ cells that would account for the enhanced production of IgG. The percentages of CD4⁺45RA⁺ cells and CD4⁺45RA⁻ cells in both groups were not significantly different from those of normal age-matched, non-institutionalized controls (CD4⁺45RA⁺ cells; 32.5 $\pm$ 11.5% and CD4⁺45RA⁻

Fig. 1 Relationship between serum levels of IgG and percentages of peripheral blood CD3⁺4⁺8⁺ double negative lymphocytes. The linear regression line is indicated, the coefficient of correlation was 0.46872 and the slope was significantly different from zero (*P* < 0.05)

Fig. 2 Percents of positivity of the CD3⁺TCR- $\alpha\beta$ ⁺ cells (a), CD3⁺4⁺8⁺ cells (b), CD3⁺TCR- $\gamma\delta$ ⁺ cells (c), and CD4⁺8⁺TCR- $\alpha\beta$ ⁺ cells (d) were compared for the normal IgG and elevated IgG groups. As shown in (a), the high IgG group had fewer CD3⁺TCR- $\alpha\beta$ ⁺ cells. This compensated for the increase in CD3⁺4⁺8⁺ T-cells in the former group (b). The CD3⁺4⁺8⁺ T-cells consisted of CD3⁺TCR- $\gamma\delta$ ⁺ (c) and CD4⁺8⁺TCR- $\alpha\beta$ ⁺ (d) cells, both of which appeared in larger numbers in the high IgG group. Data are means $\pm$ SDs of the positive cells for each group

Fig. 3 Typical representations of the expression of TCR on T-cells. CD3⁺4⁻8⁻ cells (a), CD3⁺TCR-γδ⁺ cells (b), and CD4⁻8⁻TCR-αβ⁺ cells (c)

cells; $15.3 \pm 3.0\%$). The CD8⁺ (suppressor/cytotoxic) cells were also functionally subdivided into CD8⁺11b⁺ (suppressor) and CD8⁺11b⁻ (cytotoxic) cells [11], but the former cells, which should act as effector suppressor cells in antibody production, were not decreased in the high IgG group in spite of the possible enhancement of IgG synthesis in these patients. In addition to the T-cells, natural killer cells identified by the CD16 and CD56 antibodies were retained equally by both IgG groups. These findings are compatible with those of previous studies in which abnormalities of lymphocyte subsets in severely handicapped children were not clearly shown.

Fig. 4 Schematic drawings of the constitution of the CD3⁺ T-cells in normal IgG and high IgG patients. (DN double negative)

In contrast, a positive correlation was found between the percentages of CD3⁺4⁻8⁻ cells (called double negative T-cells) and serum IgG levels in all 21 patients (Fig. 1). Furthermore, this subset showed clear differences between the two groups (Fig. 2): the high IgG group expressed a statistically significant increased percentage ($7.9 \pm 3.2\%$) of CD3⁺4⁻8⁻ cells when compared with the normal IgG group ($5.5 \pm 2.1\%$). This agrees with the finding that CD3⁺TCR-αβ⁺ cells, most of which are single positive, were decreased ($63.7 \pm 10.7\%$ vs. $71.9 \pm 8.3\%$), this decrease being compensated for by an increase in CD3⁺TCR-γδ⁺ cells, most of which are double negative ($5.7 \pm 2.5\%$ vs. $3.6 \pm 1.5\%$). The CD3⁺TCR-γδ⁺ cells of several of the patients in this study expressed negligible CD8 (data not shown) which the TCR-γδ⁺ cells in murine intestinal epithelium do [14]. The majority of the CD3⁺TCR-γδ⁺ cells present in the high IgG patients should be double negative T-cells. This was verified because the percentages of CD3⁺4⁻8⁻ cells were similar to those of CD3⁺TCR-γδ⁺ cells plus CD4⁻8⁻TCR-αβ⁺ cells in each group. In our study, the high IgG group expressed an increased number of CD3⁺TCR-γδ⁺ cells, about 66% of the double negative T-cell population. The other 33% of the double negative T-cells were CD4⁻8⁻TCR-αβ⁺ cells which were also increased in the high IgG group ($2.8 \pm 1.7\%$ vs. $1.4 \pm 0.8\%$). Thus the two types of subpopulation that make up double negative cells were more frequent in the peripheral blood of the high IgG patients than in that of the normal IgG patients. Representative phenotypic profiles of the CD3⁺4⁻8⁻ cells (patient 20), CD3⁺TCR-γδ⁺ cells (patient 20), and CD4⁻8⁻TCR-αβ⁺ cells (patient 12) are shown in Fig. 3.

Discussion

Severely handicapped children are easily infected by micro-organisms that cause upper and lower respiratory tract infections. Repeated aspirations and an impaired pulmonary clearing mechanism contribute to their vulnerability. Moreover, malnutrition, medication, and long-term hospitalization may inhibit the development of a normal immune defence. However, many handicapped children have increased serum IgG levels in the absence of an active infectious disease and of anti-nuclear antibody in their sera and immunological studies have led to conflicting results [28, 37, 44]. This may be due to individual differences in the subjects studied. Underlying disease, daily activity, and medications may have affected the results of previous studies. We selected 21 patients and divided them into two groups on the basis of their serum IgG levels. These two groups were made up of age-, disease-, and medication-matched patients, all of whom were bedridden and were being tube fed. Although phenytoin has been reported to decrease Ig levels [41] and to depress cell-mediated immunity [15, 18, 40], serum levels of IgG were not significantly correlated with the length of time or with dosage of phenytoin in our study, which is compatible with a previous study [37]. All were clinically free of active infectious diseases when examined. Furthermore, all the subjects studied had had bacterial infections that included bronchitis or pneumonia, two or three times a year.

Double negative T-cells were more frequent in the blood of the high IgG patients than in those of normal IgG patients (Table 2, Fig. 1, 2). T-cells bearing TCR- $\gamma\delta$ and those bearing TCR- $\alpha\beta$ constituted the double negative cell population in the elevated IgG group (shown schematically in Fig. 4).

The functional significance of the double negative cells has yet to be fully characterized. Human beings with rheumatoid arthritis and immunodeficiencies have increased numbers of TCR- $\gamma\delta$ cells in their peripheral blood [19, 26, 35]. Increased numbers of TCR- $\gamma\delta$ cells have been found in necrotic areas of Kikuchi and tuberculous lymphadenitis [12] as well as in granulomatous lesions of leprosy and leishmaniasis [22]. Increased numbers of TCR- $\gamma\delta$ cells have also been reported in intra-epithelial lymphocytes taken from the gut of patients with coeliac and inflammatory bowel diseases [42, 45]. In our experience, TCR- $\gamma\delta$ cells were increased in the peripheral blood in patients with chronic inflammatory bowel disease, which is compatible with the previous report [16]. In *in vitro* experiments, double negative T-cells that expressed TCR- $\gamma\delta$ predominantly proliferated in response to bacteria [1], *Mycobacterium* [27], and 65 kDa mycobacterial heat shock protein (Hsp65) [5]. These results suggest that TCR- $\gamma\delta$ T-cells function as the major component of primary defence mechanisms. As stress proteins are well conserved in the phylogeny from prokaryocytes to eukaryocytes [20, 47], it is reasonable that TCR- $\gamma\delta$ T-cells are also capable of responding to heat shock protein

of self cells induced by high temperature [34]. These findings suggest that bacterial infection may induce self heat shock protein because of the high temperature they bring about and may amplify the proliferation of TCR- $\gamma\delta$ T-cells in the patients of our high IgG group.

Like the TCR- $\gamma\delta$ T-cells in human auto-immune diseases and immunodeficiencies, CD3⁺4⁻8⁻ TCR- $\alpha\beta$ ⁺ T-cells were increased in the spleens of auto-immune mice [17] and in the peripheral blood of patients with systemic lupus erythematosus [38] and those with immunodeficiencies [46]. Investigations of double negative T-cells have related these cells to the major causes of auto-immune diseases. Results of one study suggested that the helper function is retained by double negative TCR- $\alpha\beta$ ⁺ cells with respect to immunoglobulin production [31]. In our *in vitro* experiments, double negative T-cells failed to help immunoglobulin synthesis in the presence of pokeweed mitogen (data not shown). These results may not suggest the lack of helper activity in double negative T-cells, because double negative T-cells may require the presence of other T-cells to express their helper function.

In addition to the helper function, increased TCR- $\alpha\beta$ ⁺ cells of the double negative phenotype in the livers of mice injected with bacteria are self-reactive and are suggested to be beneficial for the surveillance of autologous bacteria-infected cells [2]. Moreover, in human beings, evidence has been presented that CD4⁺8⁻TCR- $\alpha\beta$ ⁺ cells are activated by bacterial superantigens [33]. As superantigens may initiate auto-immune disease [13, 30], double negative lymphocytes activated by superantigens in severely handicapped children may reflect the activated immune status. The activated immune system will predispose to the auto-immune disease, although none of the patients in our study were positive for serum anti-nuclear antibody despite a high incidence of systemic lupus erythematosus in patients receiving anticonvulsants [4, 39].

Taking into account these findings, the increase of double negative T-cells of both TCR types found in the high IgG patients may reflect activation of the body's defence mechanism against a micro-organism which is infecting the person. Thus the increase in double negative T-cells that have the potential to react with microbial superantigens may assist B-cells to produce immunoglobulins, as evidenced by the superantigens that mediate T-helper-B-cell interactions [13].

Furthermore, the fact that three patients in the high IgG group died after the completion of this study suggests that an increase in double negative cells may herald future aggravation of a patient's condition.

The increase in double negative cells seen in high IgG patients offers a new immunological dimension by which to investigate the defence mechanisms and the development of auto-immune disease-like phenomenon of severely handicapped children.

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