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REACTIVITY OF NEW ADHESION MOLECULES ON LYMPHOCYTES FROM PATIENTS WITH CHRONIC GRAFT VERSUS HOST DISEASE*

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Reaction patterns of the 7th Human Leukocyte Differentiation Antigen Workshop blind panel adhesion molecules were studied on CD3/CD4, CD3/CD8, CD3/TCRγδ double positive T cells from peripheral blood of patients with chronic graft versus host disease (n=8) and healthy controls (n=4). Reactivity of 14 adhesion antibodies was tested by three-colour immunophenotyping. The mean proportion of CD3+ T cells (69±19%), CD3/CD8++ (31±13%) and CD3/TCRγδ++ (4±2%) T sub-populations of patients were comparable with the healthy controls. However, the mean percentage of CD3/CD4++ T cell subset in patients (14±12%) proved to be significantly decreased in comparison with the normal control value (34±16%) presumably due to secondary immunodeficiency. The workshop antibodies proved to be reactive with three T cell subsets expressing the examined antigens. Based on the results of the adhesion molecule workshop new CD categories have been introduced: CD156b as a transmembrane protein, CD167a as an epithelial tyrosin kinase receptor, CD168 as a receptor for hyaluronan mediated motility (RHAMM) and CD171 as a co-stimulatory adhesion molecule. There were significant differences in the expression of the CD167a and CD156b antigens on the CD3/CD4++ subset between the samples of patients compared with the controls characterizing the CD4+ T lymphocyte subpopulation in chronic graft versus host disease.

Keywords: adhesion molecule, allogeneic transplantation, chronic GVHD

Introduction

Blood cells developing in the bone marrow require adhesive interactions with stromal reticular cells, specialized macrophages and with other defined components of the extracellular matrix. Receptors that are known to participate in these interactions

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include certain integrins, selectins, members of the immunoglobulin superfamily, extracellular matrix receptor type III (CD44), and this list may be expected to grow [1]. The preferential distribution of lymphocyte subsets in tissues is attributed to a selective lymphocyte-endothelium interaction during entry. However, proliferation and death within the tissue, and exit from the tissue might also play a role [2]. Haematopoietic stem cell transplantation (HSCT) is used to restore normal haematopoiesis following myeloablative and immunosuppressive chemotherapy or chemo-radiotherapy [3]. In allogeneic HSCT the newly developed donor derived neutrophils and monocytes seem to be able to carry out their most important functions as soon as they are generated. Such acquisition of full normal function, however, does not apply to T and B lymphocytes, some of whose functional activity is depressed [3]. The most apparent abnormalities in cellular reconstitution are seen in chronic graft versus host disease (GVHD) [3,4]. Chronic GVHD occurs in 25-75% of patients undergoing allogeneic HSCT, despite GVHD prophylaxis. Chronic GVHD has clinical and pathogenic characteristics similar to autoimmune diseases, such as scleroderma and Sjogren's syndrome. The damage in the newly reconstituted immune system induced by many factors may contribute to both the immunodeficiency and autoimmunity characterizing chronic GVHD [3, 4].

In case of chronic GVHD not only the development and function but traffic, homing and preferential tissue distribution of T cells are supposed to be altered [4, 5]. Chronic GVHD can seriously complicate allogeneic HSCT, however, its graft versus leukaemia (GVL) effect is important for the disease-free survival of the patients [6, 7]. Although pathomechanism of chronic GVHD is not yet clear, variation of theories exist, including the role of adhesion molecules. Among the adhesion structures ICAM-1, VCAM-1, ELAM-1 and HLA-DR expression was examined and found to be increased in skin and gastrointestinal tract cell samples from allogeneic HSCT recipients with chronic GVHD, but lack of adhesion molecule expression could have seen in the liver [8]. TCR$\gamma\delta$ bearing lymphocytes were also examined in patients with chronic GVHD demonstrating elevated percentage in the peripheral blood (PB) [9].

Our working hypothesis for detection of adhesion molecule expression on PB T lymphocytes in patients with chronic GVHD has been aimed to detect the reactivity of antibodies to determine their specificity and better characterising the T cell sub-populations in this peculiar autoimmune-like disease.

Patient and methods

Eight patients (6 male and 2 female) underwent an allogeneic HSCT from HLA-identical related donors were examined. Diagnosis of all the patients was chronic

myeloid leukaemia in first chronic phase. Mean age of the patients was 42.7±7.9 years. Six out of them received bone marrow and 2 patients peripheral blood stem cells, respectively. Clinical and laboratory data of the patients are summarized in Table I.

Table I

Clinical and laboratory data of chronic GVHD patients

Number of patient	Age (yrs)	Disease	Donor	Conditioning Regimen	Chronic GVHD therapy	WBC (G/L)	Ly (G/L)
1	37	CML	HLA-id. rel.	TBI/Cy	P+CSA+My.M.	2.16	0.8
2	45	CML	HLA-id. rel.	TBI/Cy	P+CSA+My.M	4.81	0.7
3	28	CML	HLA-id. rel.	TBI/Cy	P+CSA+My.M	4.28	0.4
4	44	CML	HLA-id. rel.	TBI/Cy	P+Azath	6.4	2.3
5	38	CML	HLA-id. rel.	DBM/Ara-C/Cy	P+Azath.	3.0	0.7
6	49	CML	HLA-id. rel.	DBM/Ara-C/Cy	P+Azath	6.9	0.4
7	52	CML	HLA-id. rel.	DBM/Ara-C/Cy	Azath.+CSA	3.8	1.8
8	49	CML	HLA-id. rel.	DBM/Ara-C/Cy	P+CSA	6.9	1.7

CML: chronic myeloid leukaemia, TBI: total body irradiation, Cy: cyclophosphamide, P: prednison, CSA: cyclosporine A, DBM: myelobromol, Ara-C.: cytosin arabinoside, Azath.: azathioprine, My.M.: mycophenolate mofetil, DBM/Ara-C/Cy: new conditioning regimen for transplantation of CML [5].

Heparinised PB was obtained - with informed consent - from the patients suffered from chronic GVHD post-transplant. Control samples were obtained from 4 healthy age matched persons. After the Ficoll-Hypaque mononuclear cell separation, the measurements were carried out by Becton-Dickinson FACS Calibur flow cytometer using three-colour immunofluorescence method [10, 11].

Antibodies: CD3 PerCP (Becton-Dickinson, San Jose, CA); CD8 FITC/CD4 PE (Dako A/S, Denmark); TCRγδ (Becton-Dickinson, San Jose, CA); a-mouse-IgG F(ab')2-FITC (Dako A/S, Denmark); 7th Human Leukocyte Differentiation Workshop (HLDA, Harrogate, UK, 2000) unclustered adhesion panel antibodies: 70007, 70187, 70262, 70263, 70344, 70440, 70442, 70443, 70497, 70498, 70620, 70632, 70633, 70700.

Three colour immunofluorescence [10]: Briefly: 1.) 5×10^5 separated mononuclear cells in 50µl PBS and 1:100 diluted/undiluted supernatants Workshop antibody were incubated at 4°C, for 20 minutes. Twice washes with PBS. 2.) Fifty µl a-mouse-IgG F(ab')2-FITC was added and incubated for 20 min. at room temperature. Once PBS. 3.) Twenty µl CD3PerCP/CD4PE, CD3PerCP/CD8PE, CD3PecCP/TCRγδPE antibodies were added, incubated for 15 minutes at 4°C. Twice

PBS. 4.) Fixation: 200 µl 1% PBS-formaline. Samples were diluted with PBS before measurement.

Controls: (1) Cell autocontrol without labelling; (2) CD45 panleukocyte marker positive control for lymphocyte-gate with CD14 excluding monocytes; (3) Isotype control antihuman IgG1/G2a; (3) Anti-mouse FITC (second antibody); (4) Double CD3PerCP/CD4PE, CD3PerCP/CD8PE, CD3PerCP/TCRγδPE were used for detection of T cell subpopulations and controls for triple labelling.

Flow cytometry: Measurement was carried out by Becton-Dickinson FACS Calibur flow cytometer. The analysis of mononuclear cells (MNC) was performed by gating for lymphocytes using forward and orthogonal light scattering (min. number of 2000 events in the gate). Data acquisition was carried out by CellQuest Macintosh HD. Analyses were performed by WINLIST software.

Statistical method: The differences were tested by Student's t-test and $p<0.05$ was considered as statistically significant.

Results

The reactivity of workshop adhesion panel MoAbs was studied on CD3/CD4, CD3/CD8, CD3/TCRγδ double positive T cells from PB by three-colour fluorescence method. The mean proportions of CD3+ T cells (69±19%), the CD3/CD8++ (31±13%) and CD3/TCRγδ++ (4±2%) T cell subsets were comparable with the controls (CD3+ T cells: 63±16%, CD3/CD8++: 31±7%, CD3/TCRγδ++: 6±4%). However, the mean proportion of CD3/CD4++ cells proved to be lower (14±12%) in the patients than in the controls ($p<0.03$) (Fig. 1).

The reactivity of the 14 unclustered MoAbs was finalized during the 7th HLDA Workshop (Harrogate, UK, 2000). New CD antigens were defined and characterized as CD156b (transmembrane protein, releases soluble TNF-alpha and TGF-alpha), CD167a (epithelial tyrosin kinase receptor), CD168 (receptor of hyaluronan mediated motility = RHAMM), CD171 (co-stimulatory adhesion molecule) (Table II).

The binding of adhesion panel antibodies to the PB CD3+ T lymphocytes is shown in Table III. All MoAbs proved to be reactive both with patients' and control lymphocytes. However, expression of CD156b, CD167a and CD168 antigens was significantly higher in the patient's group.

The adhesion molecule CD156b has been expressed by a small group of CD3+ T cell subsets in chronic GVHD patients: CD3/CD4/CD156b triple positive (+++) cells: 9±9%; CD3/CD8/CD156b+++ cells: 8±11%; and CD3/TCRγδ/CD156b+++ cells 11±12%, respectively (Table IV, a, b, c). However, the percentage of CD3/TCRγδ

/CD156b triple positive cells proved to be significantly higher in the patients than in the controls ($p<0.04$).

Table II

New CDs of adhesion antigens

New CD Antigens	Workshop adhesion MoAb	Characterisation
CD156b	70620	Zinc metalloprotease with broad reactivity; TNF converting enzyme
CD167a	70262, 70263	tyrosin-kinase receptor; wide expression on normal and transformed epithelial cells
CD168 (RHAMM)	70497, 70498, 70632, 70633	Receptor for hyaluronan mediated motility
CD171	70700	Co-stimulatory adhesion molecule

Table III

Reactivity of workshop adhesion panel MoAbs on PB CD3+ T lymphocytes (mean±SD)

Workshop adhesion panel MoAbs	Chronic GVHD (n=8) (%)	Control (n=4) (%)	Statistical significance (p value)
70620 (CD156b)	11±12	3±5	0.04*
70262 (CD167a)	17±16	4±6	0.004**
70263 (CD167a)	18±16	1±2	0.01*
70497 (CD168, RHAMM)	11±12	4±5	0.06
70498 (CD168, RHAMM)	11±12	4±5	0.05*
70632 (CD168, RHAMM)	12±13	4±5	0.04*
70633 (CD168, RHAMM)	11±14	4±6	0.09
70700 (CD171)	12±13	4±5	0.07
70007	10±10	3±4	0.01*
70187	6±8	3±3	0.006**
70344	99.8±0.3	99.0±0.9	0.1
70440	9±8	2±4	0.01*
70442	11±12	2±2	0.009**
70443	14±15	3±5	0.005**

* $p<0.05$, ** $p<0.01$

The expression of CD167a molecule showed significant increase on CD3/CD4 and CD3/TCRγδ double positive cells in the patients compared with the controls ($p<0.0015$-0.04 and $p<0.01$), (Table IV, a, c).

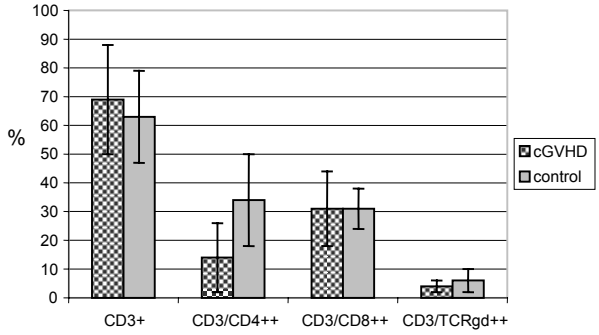

Fig. 1. The mean proportions of CD3+ T subsets in patients with chronic GVHD and controls (mean ±SD)
* p<0.03, TCR gd: T cell receptor gamma/delta

Table IVa

Reactivity of workshop adhesion panel MoAbs on PB CD3+/CD4+ T subpopulation in healthy controls and patients with chronic GVHD (mean ±SD)

Workshop adhesion panel MoAbs	Chronic GVHD (n=8) CD3+/CD4+ (%)	Control (n=4) CD3+/CD4+ (%)	Statistical significance (p value)
70620 (CD156b)	9±9	1±1	0.07
70262 (CD167a)	14±8	1±1	0.015*
70263 (CD167a)	17±13	1±1	0.04*
70497 (CD168, RHAMM)	11±11	1±0.9	0.12
70498 (CD168, RHAMM)	14±17	1±0.8	0.16
70632 (CD168, RHAMM)	10±12	1±1	0.17
70633(CD168, RHAMM)	11±12	1±0.9	0.14
70700 (CD171)	8±8	2±2	0.17
70007	8±6	1±0.4	0.052
70187	10±6	1±0.4	0.02*
70344	100±0.3	99.3±0.5	0.02*
70440	9±8	1±0.8	0.06
70442	12±8	1±0.8	0.03*
70443	12±8	1±0.7	0.02*

* p<0.05

There were 4 RHAMM molecules (CD168) examined in our study and expressed on the minor fraction of CD4 positive (11.5±13%) and CD8 positive cells (6.5±7.75%) in chronic GVHD patients, respectively (Table IV. a, b). Higher mean percentage of CD168/CD3/TCRγδ triple positive subpopulation measured in the

patients may be due to the relatively small amount of this subset (Fig. 1 and Table IV c).

Table IVb

Reactivity of workshop adhesion panel MoAbs on PB CD3+/CD8+ T lymphocytes in healthy controls and in patients with chronic GVHD (mean ±SD)

Workshop adhesion panel MoAbs	Chronic GVHD (n=8) CD3+/CD8+ (%)	Control (n=4) CD3+/CD8+ (%)	Statistical significance (p value)
70620 (CD156b)	8±11	3±3	0.45
70262 (CD167a)	11±19	3±3	0.41
70263 (CD167a)	12±19	3±3	0.35
70497 (CD168, RHAMM)	5±5	4±5	0.76
70498 (CD168, RHAMM)	6±7	3±3	0.4
70632 (CD168, RHAMM)	9±11	3±4	0.33
70633 (CD168, RHAMM)	6±8	4±5	0.6
70700 (CD171)	7±9	3±3	0.52
70007	6±10	2±3	0.38
70187	8±12	2±2	0.36
70344	100.0±0.5	99.2±0.5	0.1
70440	3±3	1±2	0.18
70442	3±3	2±2	0.45
70443	7±10	2±2	0.35

* $p<0.05$

Mean expression of CD171 was measured on all CD3+ subsets (CD3/CD4++: 8±8%, CD3/CD8++: 7±9%, CD3/TCRγδ: 12±13%) of PB T lymphocytes from chronic GVHD patients and proved to be not significant compared with the controls.

The expression of three still unclustered antigens (70187, 70442, 70443) was significantly higher on the CD3/CD4++ T subset in the patients compared with the controls (Table IV a). Furthermore, expression of five still unclustered antigens (70007, 70187, 70440, 70442, 70443) was also significantly increased on patient's CD3/TCRγδ cells. Expression of MoAb 70344 was very high in all the groups of PB T lymphocytes in patients and controls (Table IV a, b, c).

Discussion

In this study we examined the expression of adhesion molecules (as workshop unclustered panel antibodies) on PB T cells in patients with chronic GVHD and healthy controls to characterize their specificity and disease association. Eight out of 14 MoAbs have been grouped into a newly defined cluster of designation (CD) representing

adhesion molecules with different functions. Six MoAbs are still unclustered, but evaluated in this study.

Table IVc

Reactivity of workshop adhesion panel MoAbs on PB CD3+/TCRγδ T lymphocytes in healthy controls and in patients with chronic GVHD (mean ±SD)

Workshop adhesion panel MoAbs	Chronic GVHD (n=8) (%)	Control (n=4) (%)	Statistical significance (p value)
70620 (CD156b)	11±12	5±8	0.04*
70262 (CD167a)	17±16	8±10	0.004**
70263 (CD167a)	18±16	8±8	0.01*
70497 (CD168, RHAMM)	11±12	7±7	0.06
70498 (CD168, RHAMM)	11±12	7±8	0.05*
70632 (CD168, RHAMM)	12±13	7±8	0.04*
70633(CD168, RHAMM)	11±14	7±9	0.09
70700 (CD171)	12±13	7±9	0.07
70007	10±10	5±7	0.01*
70187	6±8	4±6	0.006**
70344	99.8±0.3	98.6±1.6	0.1
70440	9±8	7±6	0.01*
70442	11±12	3±3	0.009**
70443	14±15	6±7	0.005**

*p<0.05, ** p<0.01

According to the new CD classification defined by the 7th Workshop on Differentiation Antigens (Harrogate, UK, 2000) MoAb 70620 tested in our experiments belongs to the CD156b cluster of designation (Table II). It is characterized as a multi-domain, transmembrane protein that includes a Zn-dependent protease domain in the extracellular portion. It releases the soluble forms of tumor necrosis factor (TNF) and transforming growth factor-alpha (TGFalpha) from cells [12]. The adhesion molecule CD156b was expressed on the small proportion of CD3+ T cell subpopulations in chronic GVHD patients (Table III). Among the three T subsets from the patient samples statistically significant difference has been determined in the expression of CD156b antigens on the CD3/TCRγδ++ subpopulation compared with the controls.

70262 and 70263 MoAbs examined in this study proved to belong to the new CD167a cluster of Abs and corresponding antigens. CD167a is a receptor tyrosine kinase widely expressed both in normal and transformed epithelial cells and being activated by various types of collagen. CD167a belongs to a subfamily of tyrosine kinase receptors and is thought to be important in maintenance of morphology, cytoskeletal organisation and the ability to align with other cells during aggregation. CD167a plays a role in cell-cell contact and in the adhesion signalling pathways [13].

In our study the two tested MoAbs (70262,70263) related to this family showed significantly higher reactivity on the examined CD3+ T cells, and the CD4 and TCRγδ++ subpopulations (Tables III and IV a, c). CD168 (MoAbs 70632, 70633, 70497, 70498) represents a receptor for hyaluronan mediated motility (RHAMM). These newly clustered molecules indicate hyaluronan (HA)-binding receptor that participates in HA-dependent motile behaviour of thymocytes, lymphocytes, haematopoetic progenitor cells and malignant B lymphocytes. Both surface and intracellular forms of RHAMM can be detected in various cell types. Intracellular RHAMM predominates in adherent cells. 10-30% expression could have seen on a subset of human thymocytes [14]. Among the PB T cells 10-30% is known to express RHAMM [15]. On activation, normal B and T cells transiently upregulate surface RHAMM [16]. There were 4 RHAMM molecules examined in our study showing high expression on CD3+ T cells and CD3/TCRγδ++ subpopulation.

CD171 (MoAb 70700) is a newly clustered molecule in the immunoglobulin supergene family [17]. CD171 has the role for keeping the lymph node architecture during an immune response [18] and the co-stimulation of T-cell activation *in vitro* [19]. However, CD171 has also been shown to support interactions with a variety of heterophilic ligands including proteoglycans, integrins, CD9, CD24, CD56, CD142 and CD166 molecules [20]. Intermediate to low expression could have seen on human lymphoid and myelomonocytic cells, including CD4 positive cells, a subset of B-cells, monocytes, monocyte-derived dendritic cells, and follicular dendritic cells *in situ* [21]. We have also found no significant difference in the expression of CD171 between the chronic GVHD patients and the controls.

Non-clustered MoAbs (70007, 70187, 70344, 70440, 70442, 70443) also showed reactivity with PB T lymphocyte subpopulations. 70344 was the unique MoAb showing near 100% positivity on all T cell subsets examined in this study. The same positive result on control PBL suggests that 70344 molecule is present on all mononuclear cells independently of their resting or activating state. Furthermore, the mean expression of four unclustered antigens (70187, 70344, 70442, 70443) was significantly higher on the CD3/CD4++ T subset from patient samples compared to the controls.

In summary, CD156b, CD167a and CD168 (RHAMM) molecules showed significantly higher expression on CD3/CD4++, CD3/TCRγδ++ T subpopulations in the chronic GVHD patients. Presumably, the appearance of these three new CD antigens on the CD4+ and TCRγδ+ T cells might be associated with the development of the chronic graft versus host disease. Recently, the molecular basis of T cell trafficking and activation has been unravelled and various pathways controlling T cell activation following allo-HSCT have been defined. The cross-talk of different cells and

a cascade of signalling events direct and regulate the trafficking, homing and activation of T lymphocytes after HSCT [22]. Understanding their role in homing and re-population of bone marrow and blood might also be important in prevention of severe post-transplant complications including chronic GVHD.

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