

Short paper

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Expression of CR2/EBV receptors on human thymocytes detected by monoclonal antibodies*

The biologic effects of the third component of complement, C3, are mediated via receptors which specifically bind the enzymatic degradation products resulting from the cleavage of C3. One of the products, C3d, has been associated with binding to the second complement receptor CR2 (CD21). This receptor, which is identical to the receptor for Epstein-Barr virus (EBV), has been primarily found on cells of the B lineage, but not on mature T cells or other cells of erythroid or myeloid lineages. In the present investigation, we report the presence of CR2 on human thymocytes. Indirect immunofluorescence analysis employing monoclonal anti-CR2 antibodies revealed a range of thymocyte reactivity from 15% to 63% in thirteen experiments using cells of different donors. Reactivity was always greater with the monoclonal anti-CR2 (CD21) antibody HB-5 than with two other antibodies which recognize distinct epitopes on the CR2 molecule. Two-color immunofluorescence analysis indicated that the brightest of the HB-5-stained thymocytes also reacted with the monoclonal anti-CD1 antibody T6 (immature thymocyte marker) while some of the duller HB-5-staining cells reacted with the monoclonal anti-CD3 antibody Leu-4 (mature thymocyte marker). Immunoprecipitation of CR2 on thymocytes with antibody HB-5 and polyacrylamide gel electrophoretic analysis revealed a protein of 145 kDa molecular mass which is consistent with the size of CR2 found on B lymphocytes. These findings raise several questions regarding the biologic role of CR2-EBV receptor on cells of the T lineage.

1 Introduction

The third component of complement, C3, is cleaved during the activation cascade to various fragments which bind to distinct cell surface receptors [1–3]. One of these fragments, C3d, has been associated with binding to the second complement receptor CR2 (CD21) [1–4]. This receptor is present on cells of the B lineage, but absent from peripheral T cells, natural killer cells and cells of erythroid or myeloid lineages [5]. It has been reported that on B cells CR2 is identical to the receptor for Epstein-Barr virus (EBV) [6–8]. In the present investigation, we have used indirect immunofluorescence and immunoprecipitation techniques with anti-CR2 monoclonal antibodies (mAb) to demonstrate the presence of CR2 on human thymocytes.

2 Materials and methods

2.1 mAb

The mAb used in this study, and their reactivities and sources are listed in Table 1. All antibodies were purified as previously

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Abbreviations: CR2: Complement receptor type 2 EBV: Epstein-Barr virus FSC: Forward scatter mAb: Monoclonal antibody-(ies) PE: Phycoerythrin SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Table 1. Reactivity of mAb used in this study

mAb ^{a)}	Reactivity ^{b)}	Source ^{c)}
OKT6	CD1	ATCC
T6-RD1 ^{d)}	CD1	Coulter
OKT11	CD2	ATCC
64.1	CD3	Genetic Systems
Leu-4-PE ^{e)}	CD3	Becton Dickinson
Leu-12 and Leu-12-PE ^{e)}	CD19	Becton Dickinson
HB-5	CD21	ATCC
anti-B2	CD21	Coulter
OKB7	CD21	Ortho

- a) Antibodies OKT6, Leu-4, and Leu-12 are of the IgG₁ isotype; OKT11, 64.1, HB-5, and OKB7 are IgG_{2a}; anti-B2 is an IgM antibody.
b) The CD nomenclature is according to the 3rd International Workshop on Human Leukocyte Differentiation Antigens [10].
c) ATCC, American Type Culture Collection (Rockville, MD); Coulter Immunology (Hialeah, FL); Genetic Systems (Seattle, WA); Becton Dickinson (Mountain View, CA); Ortho Diagnostic Systems (Raritan, NJ).
d) T6-RD1, PE-conjugated T6.
e) Leu-4-PE, Leu-12-PE, PE-conjugated Leu-4 and Leu-12.

described [9]. Fluorescein-conjugated goat anti-mouse immunoglobulin (Ig) was purchased from Jackson Immunoresearch (West Grove, PA).

2.2 Cell source

Thymus glands, obtained from pediatric patients undergoing corrective cardiac surgery, were minced through a stainless steel screen and the cells were isolated by Ficol-Hypaque (Pharmacia, Uppsala, Sweden) fractionation [11].

2.3 Cytofluorometric analysis

One- and two-color cytofluorometric analyses were performed as previously described [9]. Briefly, one-color analysis was performed by incubating with the murine mAb of interest followed by fluorescein-conjugated goat anti-mouse Ig. In two-color analysis, the first marker was detected by indirect immunofluorescence as above, while the second marker was detected by the appropriate mAb directly conjugated to phycoerythrin (PE). The latter incubation was performed in the presence of mouse myeloma protein of the same subclass as the mAb in order to prevent any nonspecific binding [9].

2.4 Radiolabeling, immunoprecipitation and gel electrophoresis

This was performed as previously described [9]. Briefly, thymocytes were labeled with Na^{125}I in the presence of Iodo-Beads (Pierce Chemical Co., Rockford, IL). After washing, membrane lysates were prepared in 1% Nonidet-P40, pre-cleared with appropriate myeloma protein, and immunoprecipitation performed with mAb HB-5. In each step the immune complexes were collected with *Staphylococcus aureus* (Pansorbin, Behring Diagnostics, La Jolla, CA). The specific immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel (7%) electrophoresis (SDS-PAGE) under reducing conditions according to the method of Laemmli [12], and the radioactive proteins were detected by autoradiography.

3 Results

We used indirect immunofluorescence staining and cytofluorographic analysis to study the reactivity of human thymocytes to anti-CR2 mAb, HB-5, OKB7 and anti-B2. Thirteen experiments were performed using thymocytes from 8 female and 5 male donors who ranged in age from 4 months to 6 years old. The results are displayed in Table 2. Fifteen to sixty-three percent of the thymocytes reacted with mAb HB-5 while anti-B2 and OKB7 displayed much weaker reactivity (Table 2). All three antibodies are known to define distinct epitopes on CR2 [5, 13-15]. In agreement with previously published data, the

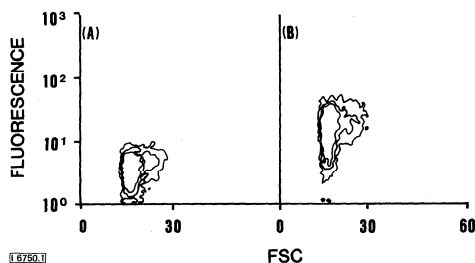


Figure 1. FSC analysis of HB-5-reactive thymocytes. Cytofluorographic analysis was performed as described in the legend of Table 2 and in Sect. 2.3. Cells were reacted with fluorescein-conjugated goat anti-mouse Ig alone (A) or with mAb IIB-5 followed by fluorescein-conjugated goat anti-mouse Ig (B). The samples were analyzed for fluorescence (vertical axis) and FSC (horizontal axis) in the FACS IV and the results are displayed as contour plots. Two replicate experiments showed identical results.

immature thymocyte marker CD1, tested with antibody OKT6, was found on 81% to 95% of the cells while anti-CD3 mAb 64.1 reacted with 53% to 76% of thymocytes [10, 16].

The lack of significant staining of the thymocyte preparations with mAb Leu-12 (anti-CD19, B cell marker) [17], and their differential reactivity with the three anti-CR2 antibodies (Table 2) exclude the possibility of contamination with peripheral B cells during the collection of the specimens. In addition, the reactivity of practically all cells with mAb OKT11 (anti-CD2, T cell marker [18]) renders unlikely contamination with thymic epithelial cells. Although epithelial cells have been previously shown to express CR2 [19, 20], they do not express CD2 [21]. Finally, the possibility that HB-5 reactivity is due to Fc binding is not very likely because in the experiments where binding of OKB7 (an mAb of the same subclass, IgG_{2a}, as HB-5) was also tested one would have expected equivalent amounts of reactivity (see exp. 1-3, 7, and 9, Table 2).

Forward scatter (FSC) analysis of HB-5-reactive thymocytes revealed two subpopulations; one with FSC ranging approximately from channel 12 to 20 (arbitrary units of FSC) and another ranging from channel 21 to 28 (Fig. 1A). While about

Table 2. Anti-CR2 reactivity of human thymocytes^{a)}

Exp. no.	Patient			Percentage of positive cells					
	Age	Sex	HB-5	CD21 anti-B2	OKB7	CD1	CD2	CD3	CD19
1	4yr	F	63	14	28	92	100	63	3
2	9mo	M	21		7	92	99	66	0
3	4mo	F	26		9	88	100	53	0
4	6yr	F	15			86	98		2
5	4yr	M	49			94	99	76	0
6	8mo	F	42			95	100	69	0
7	2.5yr	F	51	3	12	87	99		
8	2yr	M	28				96		2
9	2yr	F	43		4	92	100	59	0
10	7mo	F	28			81	99		1
11	7mo	M	54				99		2
12	2yr	M	45			81	99		3
13	2.5yr	F	44				99		1

a) Thymocytes, 1×10^6 /microtiter tray well, were incubated with mAb (20 $\mu\text{g}/\text{ml}$ of purified hybridoma IgG isolated from ascites fluids or 1:2 dilution of hybridoma culture supernatant) followed by treatment with fluorescein-conjugated goat anti-mouse Ig as described in Sect. 2.3. The cells were analyzed on the FACS IV (Becton Dickinson) using log amplification. mAb reactive with CD21 (HB-5, anti-B2, OKB7), CD1 (OKT6), CD2 (OKT11), CD3 (64.1) and CD19 (Leu-12) were used. Percentages of positive cells were calculated by computer analysis of the FACS histograms data based on negative controls that were stained with the fluorescein-conjugated second antibody alone.

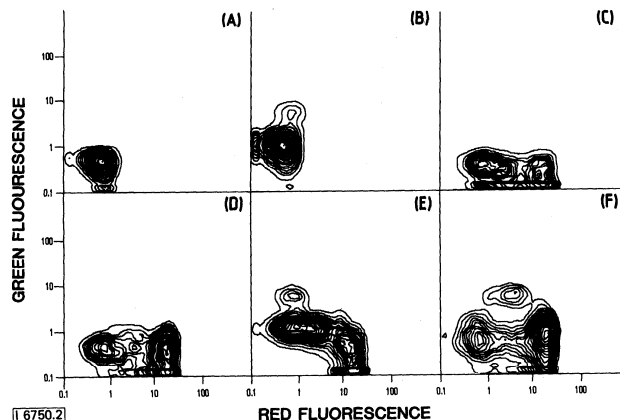


Figure 2. Two-color immunofluorescence analysis of HB-5-reactive thymocytes. Two-color staining was performed as described in Sect. 2.3. (A) Cells stained with fluorescein-conjugated goat anti-mouse Ig (green fluorescence) and PE-conjugated Leu-12 (red fluorescence) as staining controls. (B) Cells stained with HB-5/fluorescein-conjugated goat anti-mouse Ig (green fluorescence) and PE-conjugated Leu-12 (red fluorescence). (C) Cells stained with fluorescein-conjugated goat anti-mouse Ig (green fluorescence) and PE-conjugated Leu-4 (red fluorescence). (D) Cells stained with fluorescein-conjugated goat anti-mouse Ig (green fluorescence) and PE-conjugated T6 (red fluorescence). (E) Cells stained with HB-5/fluorescein-conjugated goat anti-mouse Ig (green fluorescence) and PE-conjugated Leu-4 (red fluorescence). (F) Cells stained with HB-5/fluorescein-conjugated goat anti-mouse Ig (green fluorescence) and PE-conjugated T6 (red fluorescence). Results are representative of those from two replicate experiments.

74% of the cells with the smaller FSC reacted with mAb HB-5, practically all of the larger cells were positive (Fig. 1B).

Previous phenotypic characterization of human thymocytes with mAb to lymphoid cell surface molecules has revealed several subpopulations which represent various stages of thymocyte differentiation [22-24]. Thus, mAb T6 and OKT6 (anti-CD1) react with immature thymocytes while mAb Leu-4 and 64.1 (anti-CD3) react with mature thymocytes. We wished to determine whether the HB-5-reactive thymocytes were of the mature or immature phenotype. To this end, we performed two-color immunofluorescence analysis where thymocytes were stained with mAb HB-5 and T6 or HB-5 and Leu-4. The binding of mAb HB-5 was assessed by addition of fluorescein-conjugated goat anti-mouse Ig (green fluorescence) while antibodies T6 and Leu-4 were directly conjugated to PE (red fluorescence). The results in Fig. 2 indicate that the brightest of the HB-5-staining cells also reacted with T6 (Fig. 2F), but they were negative for Leu-4 (Fig. 2E). However, some of the duller HB-5-staining cells also displayed weak Leu-4 reactivity (Fig. 2E).

In order to determine the molecular size of CR2 on thymocytes, we radiolabeled (^{125}I) cells from exp. 1 (Table 2), prepared lysates, and reacted them with mAb HB-5. The immunoprecipitates were then analyzed by SDS-PAGE under reducing conditions. This analysis revealed a single protein of 145 kDa molecular mass, a size consistent with that found on B cells (Fig. 3) [5, 8, 15, 25]. This similarity in size renders unlikely the possibility that the anti-CR2 reactivity of thymocytes represents some fortuitous cross-reactivity.

4 Discussion

This investigation provides evidence for the presence of CR2 on human thymocytes. Indirect immunofluorescence staining and cytofluorographic analysis revealed a wide range (15-63%) of thymocyte reactivity to the anti-CR2 (CD21) mAb HB 5. Thymocyte reactivity was significantly less against two other anti-CR2 mAb, OKB7 and anti-B2 which react with distinct epitopes of the CR2 molecule [5, 13-15]. It is of interest that B lymphocytes react equally well with all three mAb. For example, parallel staining of B cells along with thymocytes in exp. 1 (Table 2) revealed 98%, 90%, and 71% positive B cells with antibodies HB-5, OKB7 and anti-B2, respectively (see also [6]). This differential reactivity may be suggestive of structural differences between CR2 on B cells and that of thymocytes. However, any such differences are probably limited since the thymocyte CR2 has identical molecular size with the respective B cell receptor.

FSC analysis of thymocytes reveals two subpopulations, one a relatively small and the other a relatively large thymoblast subpopulation. Two thirds of the smaller cells were reactive with HB-5 while all of the larger ones were positive. Two-color immunofluorescence analysis revealed that the brightest of the HB-5-staining cells also expressed the immature thymocyte marker CD1. Furthermore, some duller HB-5-staining thymocytes also displayed weak anti-CD3 reactivity. Previous studies from this and other laboratories have indicated that about one quarter of thymocytes express CD1 but not CD3 differentiation markers ($\text{CD1}^+\text{CD3}^-$) while another quarter displays the reverse phenotype ($\text{CD1}^-\text{CD3}^+$) [9, 16]. In addition, one half of thymocytes co-express both differentiation markers

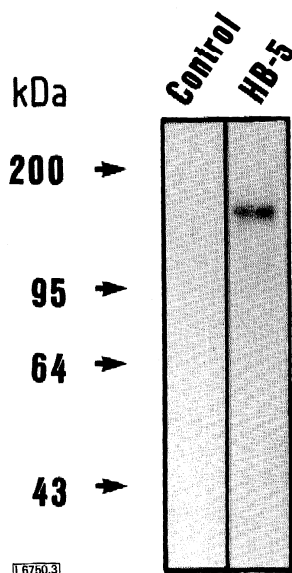


Figure 3. Molecular characterization of the HB-5-reactive protein on thymocytes. The experiment presented here was performed with cells of Exp. 1, Table 2. Surface radiolabeling, immunoprecipitation and gel electrophoresis were performed as described in Sect. 2.4. Immunoprecipitation was performed with mAb HB-5 or RPC-5 (IgG_{2a}) myeloma control. Molecular mass standards included myosin H chain (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and α -chymotrypsinogen (25 kDa). Results are representative of five replicate experiments using cells from Expts. 5, 6, 11, 12, Table 2).

(CD1⁺CD3⁺) [9, 16]. It has been proposed that the CD1⁻CD3⁺ phenotype represents functionally mature cells while the other two subpopulations represent immature or intermediate stages of thymocyte differentiation [16, 22-24]. In view of these previous studies, one possible interpretation of the staining data presented in this report could be that the CR2-bearing thymocytes represent immature cells.

In a previous study by Tedder et al. [5], thymocytes had been tested and found to be unreactive to mAb HB-5. However, it is not clear how extensive this testing was since the authors did not show any data. Furthermore, it is interesting that in the same report [5], as well as in another independent study [6], the T cell acute lymphoblastic leukemic line MOLT-4 (representative of an early stage of thymocyte differentiation) was shown to display weak reactivity with mAb HB-5. Since the cytofluorographic data reported here indicate that HB-5 binding on thymocytes is relatively weak, the possibility is raised that these previous reports simply missed such low amounts of binding. Finally, the reactivity of HB-5 with MOLT-4 suggests that CR2 may indeed be expressed at a particular stage of thymocyte differentiation.

The identity of CR2 with EBV receptors has been previously established [6-8]. Therefore, it would be intriguing to know whether EBV can bind to CR2 molecules on thymocytes and cause subsequent infection and cellular transformation. If this would be the case, thymocytic cell lines could be established and their characterization facilitated. In contrast, if EBV cannot bind to and/or transform CR2⁺ thymocytes, it would suggest the existence of functional differences between the thymocyte and B cell receptors.

The findings reported here along with previously published information raise several interesting questions regarding the biologic role of CR2. Antibodies to CR2 can elicit biologic effects on B cells including B cell growth factor-dependent proliferation and differentiation to IgM secretion [13, 26, 27]. Furthermore, the results of several studies have suggested the involvement of C3 fragments in immunologic reactions where T cells participate [1, 28-31]. In particular, Lambris and Tsokos noted that soluble C3d fragments inhibited lymphocyte proliferation induced by the mixed lymphocyte reaction or tetanus toxoid [1]. Meuth and collaborators could inhibit both mitogen or antigen-induced T cell proliferation by a C3 fragment (C3d-K) produced by cleavage with kallikrein [28]. The C3d-K fragment could also inhibit interleukin 2 synthesis in mixed lymphocyte cultures [29]. More recently, in synchronized murine B cells, Melchers et al. found that cross-linked C3d could replace α -factors essential for cell cycle progression from G₁ to S [32]. The above studies collectively suggest that CR2 may be a growth factor receptor.

Our presently ongoing studies aim at the fractionation of thymocytes into subpopulations and the utilization of anti-CR2 mAb as well as C3 fragments or synthetic peptides, representing various portions of C3 (see [4]) in order to determine their binding and also elucidate the role of this complement receptor in human thymocyte differentiation and function.

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