Brief Definitive Report

HUMAN B CELL VARIANTS IMMUNOSELECTED AGAINST A SINGLE Ia ANTIGEN SUBSET HAVE LOST EXPRESSION OF SEVERAL Ia ANTIGEN SUBSETS

By ROBERTO S. ACCOLLA

Ludwig Institute for Cancer Research, Lausanne Branch, 1066 Epalinges, Switzerland

The HLA-DR or human Ia molecules are major histocompatibility complex (MHC)-encoded polymorphic cell surface glycoproteins made up of two noncovalently linked subunits of 34–36,000 (α) and 26–29,000 (β) mol wt, respectively, which are believed to play an important role in the homeostasis of the immune system (reviewed in 1). Recent studies have shown that the human Ia molecular pool is composed of structurally distinct subsets of molecules. Some of these, like the NG1 and the NG2 subsets (2–4) are present in all individuals and constitute probably two isotypes of HLA-DR molecules. Some others of more restricted polymorphism, like DC-1 (5, 6), BR 4 × 7 (7), and I-LR1 (8) molecules, are present only in certain individuals and are believed to be coded for by genes in close linkage disequilibrium with the genes coding for the classic polymorphic HLA-DR molecules. The existence of structurally different families of Ia molecules raises the question of whether a given biological function may be related to a specific Ia subset.

One of the approaches to study the relationship between structure and function is to generate cell variants that have lost the expression of the relevant structure and then to analyze whether the absence of such structure correlates with an alteration of a specific function. As a first step toward this goal we have isolated cell variants by immunoselection using either anti-NG1 or anti-NG2 monoclonal antibodies (Mab) and complement (C). This report describes the generation as well as the phenotypic characterization of HLA-DR-negative variants selected from the human B cell line Raji.

Materials and Methods

Monoclonal antibodies. The following Mab were used in the present study: D1-12 (anti-NG1) (2–4), BT 2.2 (anti-NG2) (4), BT 3/4 (anti-DC-1) (6), and W6.32 (anti-HLA-A, B, C common) (9).

Selection procedure. Mutagenesis was performed as described by Kavathas et al. (10). Briefly, 5×10^6 Raji cells were irradiated with 300 rad and then cultured in 24-well Costar plates (Costar, Data Packaging, Cambridge, MA) precoated with irradiated mouse macrophages, at a concentration of 1×10^6/ml. After 3–4 d the cells were immunoselected by adding a saturating dose of either D1-12 or BT-2.2 Mab and C. The addition of specific anti-Ia Mab and C was repeated every 4 d over a period of 1 mo. Cells surviving the treatment were cloned under limiting dilution conditions in the presence of irradiated mouse macrophages. Representative clones from three separate immunoselection experiments were chosen for further analysis.

Flow microfluorometric analysis of stained cells. Cells were harvested from cultures in exponential phase of growth, washed twice with cold medium, and resuspended at a concentration of 5×10^6/ml. Cell suspension (0.1 ml) was incubated for 45 min at 4°C with 0.1 ml of a saturating

dose of Mab. After washing, the cells were then incubated with 0.1 ml fluorescein isothiocyanate (FITC)-coupled goat anti-mouse Ig (Nordic, Tilburg, Netherlands) for 45 min in ice. The cells were then analyzed by flow cytometry on the fluorescence-activated cell sorter (FACS II; B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA).

Radiobinding analysis. The binding test was performed as previously described (2). Briefly, 1×10^5 cells were incubated for 1 h at 4°C with 50 μl of various Mab dilutions. The amount of antibody bound was assessed by adding ^{125}I-labeled IgG rabbit anti-mouse F(ab')2.

Results and Discussion

Three cloned cell variants, RJ2.2.5, RJD1.2, and RJD1.3, derived from three independent selection experiments, were analyzed in detail in this study. The first one, RJ2.2.5, was derived after selection with the BT 2.2 Mab (anti-NG2), and the other two clones, RJD1.2 and RJD1.3, after selection with the D1-12 Mab (anti-NG1).

Fig. 1 shows the flow microfluorometric analysis of the RJ2.2.5 variant cells after cell surface staining with various Mab. It can be seen that the RJ2.2.5 cells were negative for the surface expression of both D1-12 and BT 2.2 specific epitopes. To assess whether other molecules encoded by genes within the human MHC were also affected by the immunoselection, we analyzed the expression of DC-1 molecules (5), which are normally present on the cell surface of Raji cells, as well as the expression of HLA-A, B, and C structures. Two Mab, BT 3/4 (anti-DC-1) (6) and W6.32, directed against a determinant common to HLA-A, B, and C antigens (9), were used. As shown in Fig. 1, no detectable BT 3/4-specific epitopes were found in the variant RJ2.2.5, whereas the expression of HLA-A, B, and C common determinants, as detected by W6.32 Mab, was not affected. Similar results were obtained with the other two cell variants, RJD1 and RJD1.3.

These results were confirmed by quantitative binding studies of the various Mab using ^{125}I-labeled anti-mouse F(ab')$_2$ antibodies. In Fig. 2, it can be seen that even at concentrations of Mab giving plateau binding values on the parental Raji cells, the three variants were negative for D1-12, BT 2.2, and BT 3/4 Mab. Conversely, no significant differences were observed in the amount of W6.32 specific epitopes present in the three variants as compared with Raji cells.

In addition, flow microfluorometric analysis by using FITC-coupled F(ab')$_2$ goat anti-human Ig showed no appreciable difference in the amount of surface Ig expressed by the three variants as compared with the parental Raji cells (data not shown).

Furthermore, as assessed by a rosette assay using ox erythrocytes coated with rabbit IgG anti-ox (11), both Raji cells and the three Ia-negative cell variants were found to express no detectable Fc receptors for IgG (data not shown).

When lysates from [^{35}S]methionine-biosynthetically labeled cells were immunoprecipitated by the various anti-Ia Mab used in this study and by a polyvalent rabbit anti-human Ia p 34,29 (gift of Dr F. Buchegger, Dept. of Biochemistry, University of Lausanne), no detectable Ia-specific bands were found in any of the three variants (data not shown).

Collectively, these results indicate that the three variants have concomitantly lost the expression of not only the Ia molecular subset against which they have been specifically selected, but also of other Ia subsets that are structurally distinct from the latter. Moreover, the loss of expression of Ia molecules was not accompanied by the loss of expression of other cell surface markers like HLA-A, B, C molecules or surface

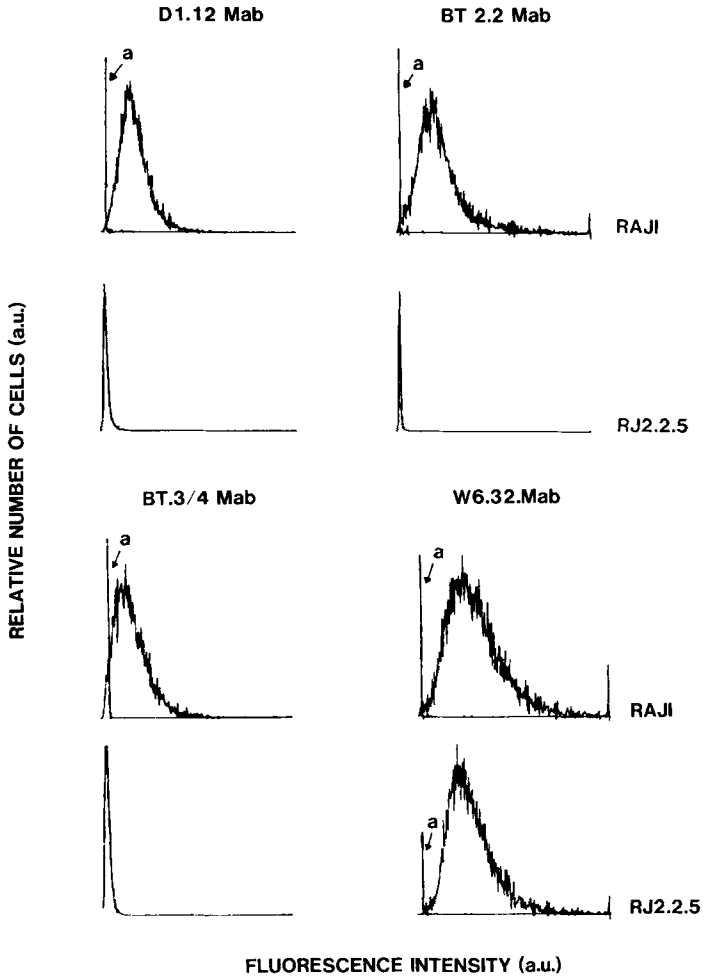

FIG. 1. Flow microfluorimetric analysis of Raji cells after immunoselection with distinct anti-Ia Mab and C. The upper series of panels (first and third) shows the flow microfluorometric pattern of the parental Raji cells when assayed for D1-12, BT 2.2, BT 3/4, and W6.32 Mab, respectively, followed by the detecting reagent, FITC-coupled goat anti-mouse Ig. The lower series of panels (second and fourth) shows the flow microfluorimetric pattern of RJ2.2.5, a cloned cell variant, when assayed with the same Mab as above. "a" represents the negative control, which in the case of D1-12, BT 2.2, and BT 3/4 Mab is the reactivity pattern of such antibodies on the Ia-negative human T cell line MOLT-4, whereas in the case of W6.32 Mab (anti-HLA-A, B, C common) is represented by the reactivity pattern of PX63 culture fluid on the two distinct cell lines. Values are expressed in arbitrary units (a.u.).

Ig. To discuss possible explanations of the above results, several considerations must be made.

The HLA-DR polymorphism is carried by the β subunit (4, 5, 12). NG1 and NG2 Ia subsets are present in all individuals irrespective of their DR phenotype. Both NG1 and NG2 subsets carry allelic polymorphism that is confined to their β subunits (5). When typed for HLA specificities, RJ2.2.5, RJD1.2, and RJD1.3 appear to have lost both DR haplotypic markers (3 and W6) present in the heterozygous parental cell line Raji as well as DC-1 markers; on the contrary, HLA typing for A, B, and C

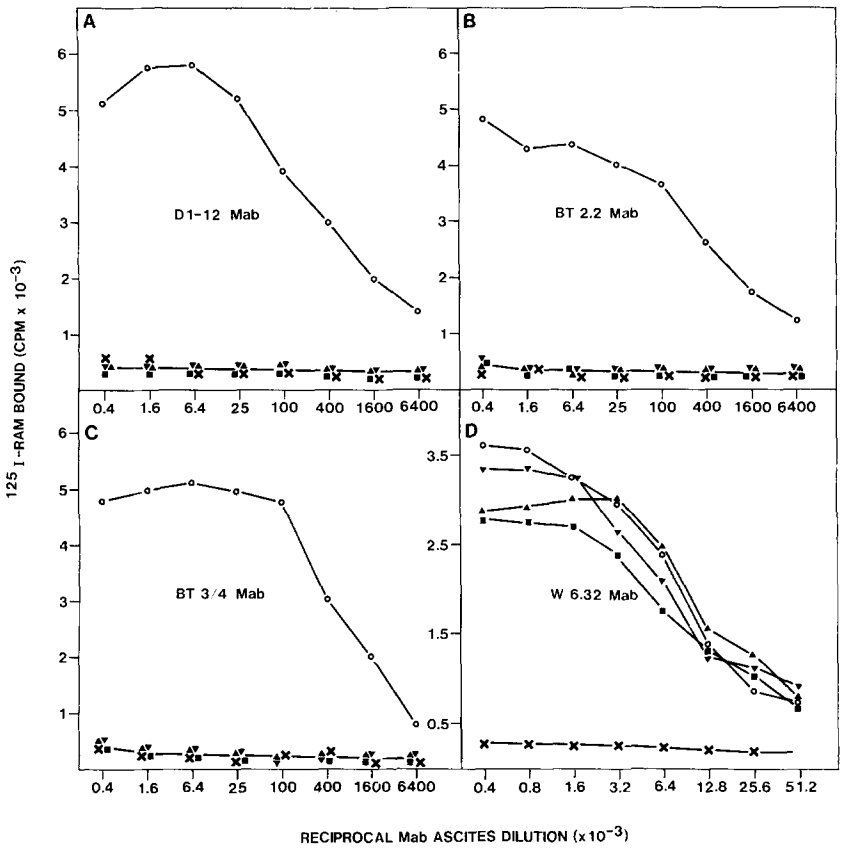

FIG. 2. Quantitation of distinct Ia epitopes and common HLA-A, B, C epitopes in immunoselected Raji cell variants. The binding test was performed as described in Materials and Methods. Each point represents the mean of duplicate values. ○: parental Raji cells; ■: RJ2.2.5 cells; ▲: RJD1.2 cells; ▼: RJD1.3 cells; ×: binding values of PX63 ascitic fluid on Raji cells which we assume as negative control. Abscissa: reciprocal Mab ascitic fluid dilution used; ordinate: total counts bound of ^{125}I-labeled rabbit anti-mouse F(ab')$_2$.

specificities was the same as that of the parental Raji cells (J. Vives, personal communication).

Taken together these results are reminiscent of those obtained by Gladstone and Pious (13), who isolated DR 1,3-negative variants by using an alloantiserum directed against only the DR 1 haplotype of a heterozygous DR 1,3 B cell line. Ionizing radiations are known to induce preferentially deletions ranging in size from intragenic deletions to losses of relevant portions of a chromosome (14). If this is the mechanism by which our variants have been generated, it is necessary to postulate a massive deletion of the different β chain coding genes located, at least for NG1 and NG2 subsets, in the two copies of chromosome 6.

The variants could be the result of a mutation/deletion of the structural gene coding for the α chain. Structural analysis has shown that NG1, NG2, and DC1 α chains differ substantially from each other (4, 6), implying a heterogeneity of the Ia α genes as well. Therefore, if mutations (or deletions) of the α genes have occurred in

the cell variants, they should have been multiple (or extensive in the case of deletions) as discussed above for the β chain-specific genes.

It might well be that other than specific Ia genes have been affected in the variants. It has been shown that biosynthetically labeled Ia molecules are associated intracellularly to a glycoprotein of 32,000 mol wt, the intermediate (In) (15) or invariant chain (16). The biological role of the In chain is not known, although it has been suggested that it might be implicated in the assembly and/or the transport of the Ia heterodimers to the cell surface. If this is the case, a mutation affecting the gene coding for the In chain would lead to an altered assembly and/or processing of the Ia subunits and therefore to the lack of detection by reagents that recognize the assembled α-β heterodimer. This possibility seems unlikely in the case of our Ia-negative variants. In fact, it has been shown that the monoclonals used in this study recognize determinants expressed on isolated β chain (2, 15). In particular, the Mab BT 2.2 is able to immunoprecipitate denaturated β chains (15) as well as the β chains synthesized in *Xenopus laevis* oocytes injected with m-RNA hybrid selected with a DRβ chain specific cDNA clone (17). Therefore, detection of newly synthesized β chains in the absence of In chain should be possible in our experimental system.

As an alternative explanation of the results obtained, we must consider (*a*) a defect of the posttranscriptional steps leading to a correct synthesis of the mRNA, specific for NG1, NG2, and DC1 Ia molecules, and (*b*) a defect of possible "regulatory" gene(s) controlling the transcription of the entire set of Ia genes and having the capacity to act in "trans" as recently proposed (18). The availability of cDNA probes of the distinct Ia subunits will greatly help in clarifying the mechanisms through which such variants have been generated and shed more light on the regulation of expression of a genetic system of high biological relevance.

Summary

Two monoclonal antibodies, D1-12 and BT 2.2, recognizing two distinct subsets of human Ia molecules, NG1 and NG2, respectively, present in all individuals irrespective of their HLA-DR phenotype, have been used to immunoselect cell variants from the lymphoblastoid cell line Raji.

Results showed that, irrespective of the monoclonal antibody used for immunoselection, the cell variants analyzed in this study had lost the expression of both D1-12- and BT 2.2-specific antigenic determinants. Moreover, the expression of antigenic determinants specific for a third family of Ia molecules, the DC-1 subset, were also lost in the cell variants. In contrast, expression of HLA A, B, and C common structures, as recognized by the W6.32 monoclonal antibody, as well as expression of surface immunoglobulins, were not affected. Possible mechanisms inducing such a coordinate loss of expression of several families of human Ia molecules are discussed.

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