TRANS-ACTING ELEMENT(S) OPERATING ACROSS SPECIES BARRIERS POSITIVELY REGULATE EXPRESSION OF MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II GENES

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In the past few years, considerable attention has been focused on a family of major histocompatibility complex (MHC)[1]-encoded human cell surface glycoproteins, the HLA class II or Ia antigens, both because of their important role in the homeostasis of the immune system, and because of their possible association with certain diseases (reviewed in 1).

The Ia molecules are heterodimeric structures made of noncovalently linked subunits of 34–36 and 24–28 kilodaltons (kD), α and β chains, respectively. Both chains are glycosylated, and are expressed on the cell surface of a variety of cells, including B cells, macrophages/monocytes, and activated T cells, as well as other normal tissues and tumor cells of different origin (reviewed in 2).

From the studies of many groups (3–11), it is now well documented that the human Ia antigens consist of a heterogeneous family of molecules with distinctive structural characteristics and various degrees of polymorphism. Molecular studies at the DNA level have established the existence of at least three class II subregions (12–15), denominated DP, DR, and DQ, respectively (16). The actual number of α and β genes residing in each subregion is not yet established, but presently available data call for at least 10–12 different functional genes per haploid genome. The products of a minimum of eight genes are expressed at the cell surface of a single B cell under the form of four distinct α-β heterodimers (17).

It is reasonable to believe that polymorphism and heterogeneity of the Ia molecules both contribute to the various functions mediated by these molecules. Furthermore, recent data from several groups (18–24) have indicated that modulation of class II antigen expression can occur in certain conditions, both in mouse and man, in various cell types, thus suggesting that the concentration of the molecules expressed on the cell surface may constitute an additional parameter to be taken into consideration for a correct understanding of the biological functions of class II antigens.

In an attempt to gain a deeper insight into the mechanism controlling the

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[1] *Abbreviations used in this paper:* 2-D PAGE, two-dimensional polyacrylamide gel electrophoresis; FACS, fluorescence-activated cell sorter; In, invariant; mAb, monoclonal antibody; MHC, major histocompatibility complex; PEG, polyethylene glycol.

expression of human Ia antigens, we have recently (25) generated Ia^- B cell variants, using gamma ray mutagenesis and immunoselection with monoclonal antibodies (mAb) and complement. Although the immunoselection was performed with monomorphic Ia-specific mAb directed against single Ia subsets, the resulting variants lost expression of all assessable class II antigens (25). Further studies (26) at the molecular level have established that the absence of expression of class II antigens was probably due to the inactivity of most, if not all, class II genes on both copies of chromosome six, since no mRNA specific for the various α and β subunits was found in the Ia^- variants. Furthermore, normal amounts of class I (HLA-A,B,C) antigens were present in these variants (25). Another molecule, the In (invariant) chain glycoprotein, which is found (27–30) associated to the Ia α-β heterodimers intracellularly, was also found (26, 30) to be expressed in the Ia^- variants, although in reduced amounts. The Ia^- phenotype of these variants is highly stable, and Ia^+ revertant clones have not been found, to date, in long term cultured cells.

By a different approach, Gladstone and Pious (31) generated variants from a DR 1,3 heterozygous B cell line by immunoselection with an alloantiserum directed against the DR1 specificity only. Some of these variants, like the 6.1.6 cells, were lacking both DR1 and DR3 specificities (31). Both DR1 and DR3 markers could be reexpressed in somatic cell hybrids between the DR^- variant and a partner cell with different DR phenotype (32). However, the high reversion frequency found (33) in the 6.1.6 cell line makes it difficult to interpret these results.

By using a similar approach, we have recently (34) found that human class II gene expression could be restored in somatic cell hybrids between the human RJ 2.2.5 Ia^- variant and the mouse Ia^+ M12.4.1 B cell lymphoma. Based on these previous results, two hypotheses have been put forward to explain the lack of expression of human class II genes in the human RJ 2.2.5 variant: (*a*), loss of a factor required to maintain or promote a high concentration of specific mRNA either at level of synthesis or, less likely, at the level of RNA stability; and (*b*), increased efficiency of a mechanism that normally counteracts overproduction of mRNA, such as a super-repressor, or the reactivation of developmentally silent extinguisher genes.

However, the fact that the RJ 2.2.5 × M12.4.1 hybrids rapidly segregated human chromosomes prevented analysis of the dominance of the RJ 2.2.5 over the wild-type phenotype. In essence, the suppression of the Ia^- phenotype upon somatic cell hybridization could either be assigned to the action of a positively-acting implementing factor contributed by the mouse partner, or to the loss of a postulated repressor-like or extinguisher function accompanying segregation of human chromosomes. In addition, the RJ 2.2.5 × M12.4.1 hybrids expressed a very low level of DQ antigen, if any (34); this lack of expression could be either attributed to some rather general property of the coexisting mouse background, or to a dichotomy of DR and DQ regulatory mechanisms. This study addresses these questions by analyzing interspecies hybrids generated by fusing RJ 2.2.5 cells with resting mouse BALB/c spleen cells. The loss of specific phenotypic markers of such hybrids in culture indicated a preferential segregation of mouse

over human chromosomes, and made it possible to better investigate the mechanism implicated in the control of class II gene expression.

Materials and Methods

Cells and Hybridization Procedures. The isolation of the human Ia$^-$ RJ 2.2.5 cell line has been previously described (25). RJ 2.2.5 cells were fused with mouse spleen cells in the presence of polyethylene glycol (PEG) (M_r 1,000; Merck & Co., Darmstadt, Federal Republic of Germany) as previously described (34). Routinely, 10^7 RJ 2.2.5 cells were mixed with 10^8 BALB/c spleen cells. After fusion, cells were seeded in 24-well Costar plates precoated with irradiated BALB/c mouse peritoneal macrophages.

Selective medium was added 24 h after cell fusion. Selective medium contained 10^{-6} M ouabain to select against unfused RJ 2.2.5 cells. Human cells are much more sensitive than rodent cells to ouabain, a steroid-like compound that inhibits the Na/K ATPase (35). Preliminary experiments showed that the ouabain concentration reducing the relative cloning efficiency of RJ 2.2.5 cells to 50% of that found in nonselective medium (LD_{50}) was 10^{-9} M, compared to a corresponding LD_{50} for rodent cells of 10^{-4} M ouabain.

Screening and Selection of Hybrids. Cells growing in selective medium were analyzed for the cell surface expression of human and murine MHC markers with the panel of mAb listed in Table I. The analysis was performed by indirect immunofluorescence on a fluorescence-activated cell sorter (FACS II; Becton Dickinson Immunocytochemistry Systems, Mountain View, CA) as previously described (25). For further studies, hybrids of interest were first sorted on the basis of positive expression of the MHC marker of choice (see Results) and then cloned by limiting dilution or by micromanipulation methods. Representative clones were chosen for detailed analysis.

Biochemical Analysis of Cell Hybrids. Hybrids were cell-surface labeled by the lactoperoxidase-catalyzed iodination method, as previously described (30). Detergent-solubilized cell extracts were reacted with specific mAb, and the resulting immunoprecipitate recovered by incubation with killed Staphylococcus Aureus Cowan I strain bacteria (Pansorbin; Pharmacia Fine Chemicals, Uppsala, Sweden). In the case of the BT 3/4 antibody (DQ-specific), a Sepharose 4 B–coupled mAb was used to purify the corresponding antigen, since this antibody does not bind to Pansorbin.

After elution, the specific immunoprecipitate was analyzed by two-dimensional gel electrophoresis according to O'Farrel et al. (36).

RNA Analysis of Cell Hybrids. Cytoplasmic RNA was prepared as described (37) and dot blotted onto hybridization transfer membranes (Gene Screen Plus; New England Nuclear, Boston, MA). Filters were hybridized to labeled cDNA probes in 50% formamide under stringent conditions, and according to the specifications of the manufacturers. The cDNA probes used in this study were: plasmid DR α-10, carrying a 1,170 basepair (bp) cDNA insert encoding the DR α chain (38); plasmid DCβ1, carrying a 1,140 bp cDNA insert encoding a DQ β chain (39); p33-22, carrying a 1,100 bp cDNA insert encoding the p33 In chain (40). DNA labelling with α-[^{32}P]dNTP was performed by nick translation according to Rigby et al. (41).

TABLE I
mAb Used in This Study

mAb	Specificity	Reference
D1-12	Human DR	3
BT 3/4	Human DQ1	4
B9.12.1	Human HLA-A,B,C	43
25.9.17	Mouse I-A^{b,d} p, q	44
S13.11	Mouse H-2K^d	42

Results

RJ 2.2.5 human Ia$^-$ cells were fused with BALB/c spleen cells in presence of PEG, and cultured in ouabain-containing medium.

Table II shows the results obtained from a representative experiment. When 10^7 RJ 2.2.5 cells and 10^8 mouse spleen cells were fused in presence of PEG, cell growth was observed in all wells (48 out of 48) after 2 wk of culture in selective medium. No cell growth was observed if PEG was omitted. Analysis of the MHC cell surface phenotype of growing cultures was then performed. The S13.11 mAb specific for murine H-2K gene products (42) revealed that 37 out of 48 cultures contained cells expressing the relevant antigen. The number of cells per culture displaying H-2K$^+$ phenotype was 10–95%. Cultures were then analyzed for the presence of human class II-specific antigens whose expression was abolished in the parental RJ 2.2.5 cells. In the experiment reported in Table II, 36 out of 48 cultures contained cells expressing DR-specific epitopes, as assessed by the D1-12 mAb (3). The number of DR$^+$ cells per culture was 3–75%. Among these cultures, 32 were also positive for H-2K antigens, thus leaving 5 cultures positive for H-2K and negative for DR markers, and 4 cultures positive for DR antigens but not for H-2K antigens.

The expression of human HLA class I antigens was also studied in this preliminary screening. All 48 growing cultures, and 100% of the cells in each culture displayed HLA-A,B,C antigens, as assessed by mAb B9.12.1 (43).

These results show that interspecies somatic cell fusion between human RJ 2.2.5 Ia$^-$ cells and normal mouse spleen cells generate hybrids in which the expression of human Ia antigens can be restored as a relatively frequent event, and in which the expression of human MHC class I antigens appears to be predominant with respect to its homologous murine counterpart.

Analysis of Human × Mouse Hybrids at Population Level. To investigate in more detail the reexpression of human Ia antigens in RJ 2.2.5 × BALB/c spleen cell hybrids, cells from a single culture expressing the highest number of both H-2K$^+$ and DR$^+$ cells were chosen for further analysis.

Hybrid cells were selected first on the basis of H-2K$^+$ phenotype, cultured in vitro for a period of 14 d, and then analyzed for the presence of H-2K and DR antigens. Although originally selected for H-2K, only 85% of the cells retained the cell surface expression of this marker. 60% of the cells were DR$^+$. This

TABLE II

RJ 2.2.5 × BALB/c Spleen Cell Hybrids Expressing Specific MHC Markers

Exp.*	Fusing agent	Growth/wells plated‡	MHC markers§			
			H-2K	DR	H-2K + DR	HLA-A,B,C
A	PEG	48/48	37	36	32	48
B	—	0/48	—	—	—	—

* In each experiment, 10^7 RJ 2.2.5 cells and 10^8 BALB/c spleen cells were mixed together and treated with either PEG (A) or nothing (B).

‡ Wells were screened for cell growth after three weeks in presence of selective medium (ouabain 10^{-6} M). Values indicate the total number of wells in which cell growth was observed/total number of wells in which the cells were plated.

§ Total number of wells in which growing cells with the specific MHC marker were found.

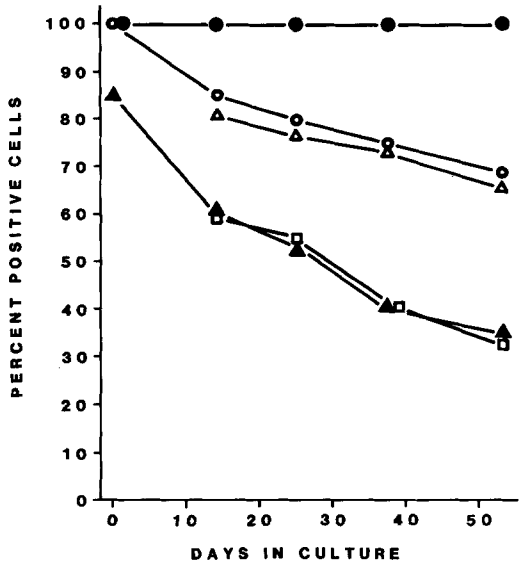

FIGURE 1. Expression of human and mouse MHC markers in RJ 2.2.5 × BALB/c spleen cell hybrids over time. Hybrids were sorted by a FACS on the basis of H-2K$^+$ phenotype, and subsequently on the basis of DR$^+$ phenotype, as described in the text. At various times (abscissa) cell hybrids were analyzed for the presence of the specific MHC markers (●, HLA-A,B,C; ○, DR; △, DQ; ▲, H-2K; □, I-A) by indirect immunofluorescence and FACS; values are expressed as percent positive cells (ordinate).

population was then sorted on the basis of its DR$^+$ phenotype. Analysis of the H-2K phenotype of the DR$^+$ cells 1 d after positive selection revealed that, again, ~85% of such cells were H-2K$^+$. This finding indicates that during the initial 14-d period of culture, a subpopulation of cells (15% in this case) lost the murine class I–positive phenotype; in this subpopulation, segregation of the mouse class I marker seemed independent of segregation of the human class II–positive phenotype.

The DR$^+$ hybrid population was kept in culture without any further selection, and analyzed for the human and murine MHC cell surface phenotype every 10–14 d over a period of 52 d. Fig. 1 shows the results of such analysis.

Human class I antigens remained constantly expressed in 100% of the population throughout the culture period. Conversely, at day 14 of culture, only 85% and 60% of cells maintained a DR$^+$ and an H-2K$^+$ phenotype, respectively. At this time, cells were also analyzed for reexpression of a different human class II marker, the DQ antigen, as well as for expression of murine I-A antigens. 78% of cells showed positive staining with the BT3/4 anti-DQ1 mAb (4), and 58% of cells were positively stained with the anti-I-A mAb 25.9.17 (44). With time, the hybrid cell population continued to lose cell surface expression of both human class II and murine class I and class II markers. At the end of the culture period, 70% and 65% of cells were still DR$^+$ and DQ$^+$ respectively, whereas 35% and 33% of cells retained the expression of H-2K and I-A antigens.

From the results presented in this section, it may appear that the number of cells expressing human DR antigens closely matched, over time, the number of

cells expressing DQ antigens, suggesting an overlap of the two populations. Similar considerations could be applied to the cells expressing mouse H-2K and I-A antigens. The rate of reduction in the number of cells expressing human class II surface antigens paralleled a reduction in number of the cells displaying both class I and class II murine markers. However, throughout the period of culture, the actual number of cells expressing human class II antigens was consistently higher than the corresponding number of cells expressing mouse MHC antigens, suggesting that reexpression of human Ia antigens in the hybrid correlated with the presence of mouse products not necessarily associated with the expression of murine MHC antigens.

Analysis of RJ 2.2.5 BALB/c Spleen Cell Hybrids at the Clonal Level. The analysis of human × mouse hybrids was then extended to clones generated by limiting dilution from the same population described in the previous section at day 38 after the DR^+ selection (see Fig. 1). 12 clones were arbitrarily chosen, amplified in culture, and characterized for their human and mouse MHC cell surface phenotype by flow microfluorometry.

Fig. 2 summarizes the results of such analysis. 100% of the cell population deriving from each clone expressed the HLA class I antigens, in agreement with the results obtained at population level. However, on the basis of their human class II and murine class I and II phenotypes, four groups of clones could be identified. Fig. 2 shows four clones, each representative of a given group; values represent the percentage of cells in the cloned population displaying a specific MHC antigen. The first group (*A*) encompassed five clones, and was characterized by the coexpression of human class II and mouse class I and II antigens in a comparable high number of cells. It must be stressed, however, that none of the clones showed expression of any one particular MHC antigen in 100% of the cells.

The second group of clones (group *B* in Fig. 2) was characterized by the presence of human DR and DQ antigens on a large proportion of cells (range 54–68%), and by a relatively much lower number of cells expressing both H-2K

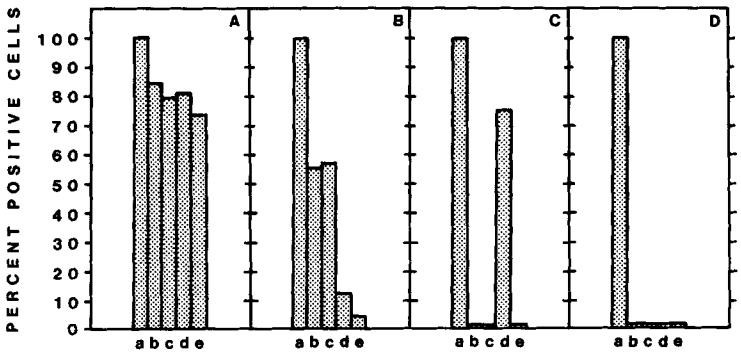

FIGURE 2. Expression of human and mouse MHC antigens in RJ 2.2.5 × BALB/c spleen cell hybrid clones. Clones were generated by limiting-dilution methods, then analyzed by indirect immunofluorescence with a FACS for the presence of specific MHC markers (a, HLA-A,B,C; b, DR; c, DQ; d, H-2K; e, I-A). Values were expressed in percent positive cells (ordinate). Four distinct reactivity patterns (*A, B, C, D*) were found in the clones studied. The histograms of four clones, each representative of a specific reactivity pattern, are shown.

and I-A antigens (range 4–19%). Within the limitation of the technique employed to count the cells positive for a given marker, the MHC phenotype of this group of clones clearly indicate the presence of hybrid cells, within a clone, which have lost expression of mouse MHC-specific determinants but still retain expression of human class II MHC determinants.

The third group of clones (group *C* in Fig. 2) included 2 clones that were characterized by the virtual absence of cell surface expression of human class II and mouse class II antigens, and by the presence in the vast majority of cells (range 65–85%) of mouse class I antigens. This finding contrasted with the results obtained at the population level, where the absolute number of H-2K$^+$ cells at any time in culture was (*a*) very similar to that of I-A cells; and (*b*) much lower than the number of human class II–positive cells.

The fourth group (Fig. 2, group *D*) comprised a single clone out of the 12 analyzed that was characterized by the virtually complete absence of cell surface expression of both human class II and mouse class I and class II antigens.

We wished to determine whether the reexpression of human class II antigens encoded by distinct subregions like DR and DQ was quantitatively similar to that found in Raji cells, the B lymphoma line from which the Ia$^-$ RJ 2.2.5 variant was generated.

Based on preliminary titration experiments, we chose saturating concentrations of the various mAb, and after staining the cells, we analyzed their MHC cell surface phenotype on the FACS. Fig. 3 shows the results obtained in a representative clone of group A (see Fig. 2 *A*) named clone A2-2. As previously mentioned, clear biphasic distribution of the staining was observed for anti-DR, anti-H-2K, and anti-I-A mAb, while the anti–human class I reagent stained 100% of the cells. The relative amount of fluorescence detected with these reagents closely paralleled that found in Raji cells (DR and HLA-class I), and in the B cell lymphoma line M12.4.1 (H-2K and I-A), respectively.

In contrast, the relative fluorescence obtained with the anti-DQ1 reagent, BT3/4, was four- to sixfold lower in the hybrid, as compared to Raji cells (Fig. 3, *DQ*). In fact, a fourfold amplification of the fluorescent signal with respect to the one used for Raji cells had to be used to obtain the histogram depicted in Fig. 3. Although a clear biphasic distribution was not appreciated, 20% of the A2-2 clone hybrid cells were included in the area that overlapped with the negative control (Fig. 3, *DQ/RJ, b*). A similar differential expression of DQ vs. DR was found in all clones of both group A and group B (data not shown) (see Fig. 2).

Phenotypic Studies in Micromanipulated Hybrid Clones. The results presented in the previous section indicated that the clonal progeny of RJ2.2.5 × BALB/c spleen cell interspecies hybrids expressed a heterogenous phenotype as far as MHC markers was concerned. To precisely assess whether the heterogeneity of expression merely reflected the preexistence of distinct hybrid clones, each one expressing stable but different phenotypes, or was due to modification within the clonal progeny of a single hybrid species, we performed the following experiment. The hybrid clone A2-2 was recloned by single-cell micromanipulation method, and among the clonal progeny, a hybrid clone was selected on the basis of the highest percentage of cells expressing both human class II and mouse

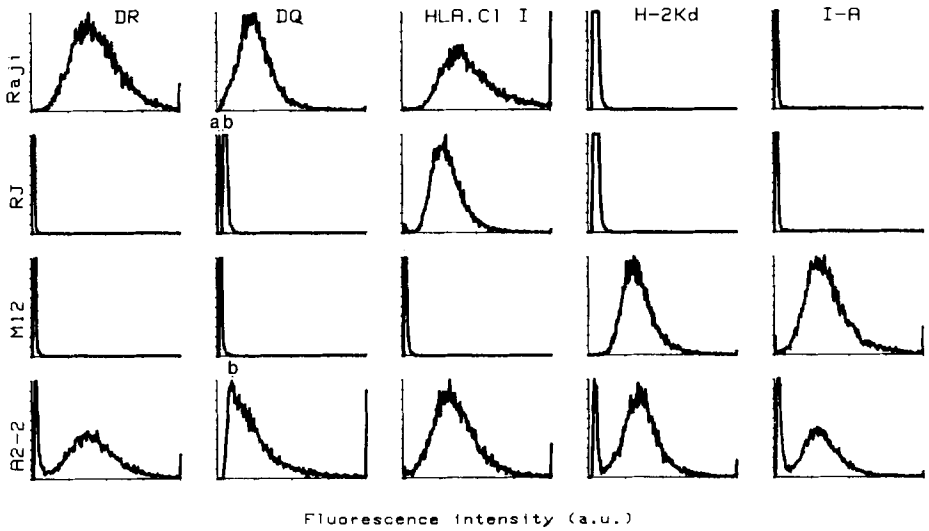

FIGURE 3. Flow microfluorimetric analysis of a human × mouse cell hybrid clone (A2-2) after staining with anti-class I and anti-class II mAb (see Table I). Each horizontal series of histograms represents a single cell line tested for the specific MHC marker listed on the top of the upper series of panels. The distinct cell lines analyzed, listed on the ordinate of the first left histogram of each horizontal series, were the human Raji cells from which the Ia^- RJ 2.2.5 cells have been derived; the RJ 2.2.5 cells (RJ); the mouse M12.4.1 BALB/c cell lymphoma (M12); the human × mouse hybrid clone A2-2 representative of the group A reactivity pattern shown in Figure 2. Values are expressed in arbitrary units (a.u.). Note that the histogram of the DQ-specific fluorescence of the A2-2 hybrid (*b*) has been obtained after a fourfold amplification of the fluorescent signal used for the Raji cells (positive control) and the RJ 2.2.5 cells (*a*, negative control). For comparison, in the panel of the negative control RJ 2.2.5, the histogram obtained after fourfold amplification of the fluorescent signal (*b*) has also been included. For further explanations see text.

class I and II antigens. This clone, named A2-2.4, displayed the following phenotype at the time of analysis: DR, 77%; DQ, 79%, H-2K, 68%; and I-A, 67%; as assessed by indirect immunofluorescence with the FACS (data not shown). Clone A2-2.4 was selected on the basis of an $H\text{-}2K^+$ phenotype, and the day after on the basis of a DR^+ phenotype. 24 h later, the MHC phenotype of the selected clone was reanalyzed to assess the number of cells expressing relevant markers, and it was found that 100% of cells did express both human class II (including DR and DQ) and mouse H-2K and I-A antigens (see Table III). At that time, single cells were picked up by micromanipulation and cultured. 61 clones deriving from the A2-2.4 hybrid clonal population were then analyzed for the cell surface expression of MHC markers.

Again, the only common feature of all clones consisted in the expression of HLA class I antigens in 100% of the cells (data not shown).

On the basis of differential expression of human class II and mouse class I and II antigens, four groups of reactivity patterns were observed (see Table III). The vast majority of clones (41 out of 61, identified as group A) expressed both human and mouse markers in a large proportion of cells. In Table III, the complete phenotype of clone c.9, a representative of group A, is reported. The

TABLE III
MHC Phenotype of Micromanipulated Clones Derived from Hybrid Clone A2-2.4

Group	Total number of clones	Representative clone	Phenotype			
			DR	DQ	H-2K	I-A
Parental	—	A2-2.4	100*	100	100	100
A	41	c.9	89	87	93	86
B	4	c.28	71	64	24	21
	2	c.14	86	87	2	1
C	4	c.29	2	1	94	1
	4	c.3	1	1	80	21‡
	4	c.24	36	NT§	91	NT
D	2	c.27	1	5	5	3

* Values are expressed in percent of positive cells detected by indirect immunofluorescence at the FACS using the mAb specific for the various MHC-markers listed in Table I.
‡ The relative fluorescence intensity detected with the anti-mouse I-A-specific reagent on this group of clones was highly reduced with respect to the one found in the parental clone A2-2.4. For further explanation see the text.
§ NT, not tested.

phenotype of group A clones closely resembled the one observed in the parental A2-2.4 population.

A second group of clones, group B in Table III, including six clones, was characterized by the predominant expression of human class II vs. mouse class I and II antigens. Among this group, four clones were identified which expressed, in 64–85% of the population, DR and DQ antigens, whereas only 20–25% of the populations showed H-2K and I-A markers. The complete phenotype of clone c.28, which is representative of this subgroup, is presented in Table III. Interestingly, the other two clones included in group B displayed DR and DQ antigens in >80% of their cells, whereas they were virtually negative for expression of both mouse class I and class II antigens. The complete phenotype of one of the two clones, c.14, is presented in the same table.

A third group of clones, group C in Table III, including 12 hybrids, displayed a peculiar phenotype characterized by the expression of H-2K antigens and by the highly reduced or even absent expression of both human and mouse class II antigens. Within this group, four clones expressed no DR, DQ, nor I-A markers (representative clone, c.29), and four clones expressed neither DR nor DQ antigens, but expressed mouse I-A antigens in a variable number of cells (range 12–35%; representative clone, c.3). It must be stressed, however, that the relative fluorescence intensity obtained with the anti-I-A-specific reagent was drastically reduced in all four clones included in this subgroup. In fact, the mean value of fluorescence of the I-A^+ cells detectable using FACS analysis was five- to sevenfold lower, with respect to the positive control, and only two- to threefold higher than the negative control (data not shown). The remaining four clones included in group C were characterized by the presence of human class II markers (DR) in only 30–35% of the cells (representative clone, c.24).

A fourth group, called D in Table III, included only 2 clones out of the 61 analyzed. These two clones were characterized by the virtual absence of expression of all human class II and mouse class I and II antigens. The complete phenotype of one of such clone (c.27) is presented in Table III.

In summary, the results described here closely resembled those obtained with clones derived by the original hybrid population, and clearly indicate that a highly homogeneous clone can give rise to heterogeneous progeny in which (*a*) concomitant segregation of mouse markers such as those of chromosome 17 can take place in the hybrids (groups B and D); (*b*) the reexpression of human class II antigens as well as reversion to human Ia^- phenotype does not correlate with the expression of products of the mouse MHC (groups B and C); (*c*) concomitant loss of expression of class II antigens of both human and mouse origin can take place without segregation of mouse chromosome 17 (group C).

Biochemical Analysis of Reexpressed Human Class II Antigens. To assess the biochemical characteristics of the human class II antigens reexpressed at the cell surface of the RJ 2.2.5 × BALB/c spleen cell hybrids, immunoprecipitation experiments followed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis were performed on cell extracts from ^{125}I surface-labelled A2-2 hybrid cells. By using mAb specific for distinct subsets of human Ia antigens such as DR (Fig. 4, *A*, *C*, and *E*) and DQ (*B*, *D*, and *F*), specific immunoprecipitates were obtained from A2-2 cells (Fig. 4, *E* and *F*) that were very similar to the corresponding ones observed in Raji cells (*A* and *B*, respectively). The resulting Ia molecules were composed of a set of acidic spots corresponding to the α chain of class II antigens, and of a set of more basic spots of lower molecular weight corresponding to the β chain of class II antigens. As previously shown (30) and confirmed here, no specific immunoprecipitates were obtained from the Ia^- RJ 2.2.5 cells (Fig. 4, *C* and *D*, respectively). Thus, by classical biochemical criteria, the Ia molecules recognized by anti-DR and anti-DQ reagents in the human × mouse cell hybrids did not differ from those expressed in the Raji B cells from which the Ia^- RJ 2.2.5 variant was generated. In agreement with the results obtained by FACS analysis, on a per cell basis, the amount of DQ-specific molecules immunoprecipitated by the BT3/4 mAb in the A2-2 hybrid (Fig. 4*F*) was significantly lower compared to that obtained in Raji cells (*B*).

Studies at mRNA Level. To finally correlate the expression of human Ia antigen in the human × mouse cell hybrids with the reappearance of RNA transcripts, we prepared total cytoplasmic mRNA from the human class II–positive clones, A2-2 (see Fig. 3) and A2-6 (a clone with a cell surface phenotype equivalent to those of group B in Fig. 2), and after immobilization on nitrocellulose filters, we hybridized the mRNA with ^{32}P-labeled cDNA probes specific for distinct subunits of various Ia subsets, as well as with a ^{32}P-labeled cDNA probe specific for the p33 invariant chain molecule. It can be seen (Fig. 5, *left*) that the DR α-specific cDNA hybridized strongly with the mRNA preparation of the A2-2 (*d*) and A2-6 (*e*) hybrid clones. Conversely, no hybridization was observed to mRNA preparations from both the RJ 2.2.5 Ia^- cells (*b*) and the mouse M12.4.1 B cell line (*c*), in agreement with our previous observations (26, 34).

Similarly, the cDNA probe specific for the β subunit of DQ antigens hybridized

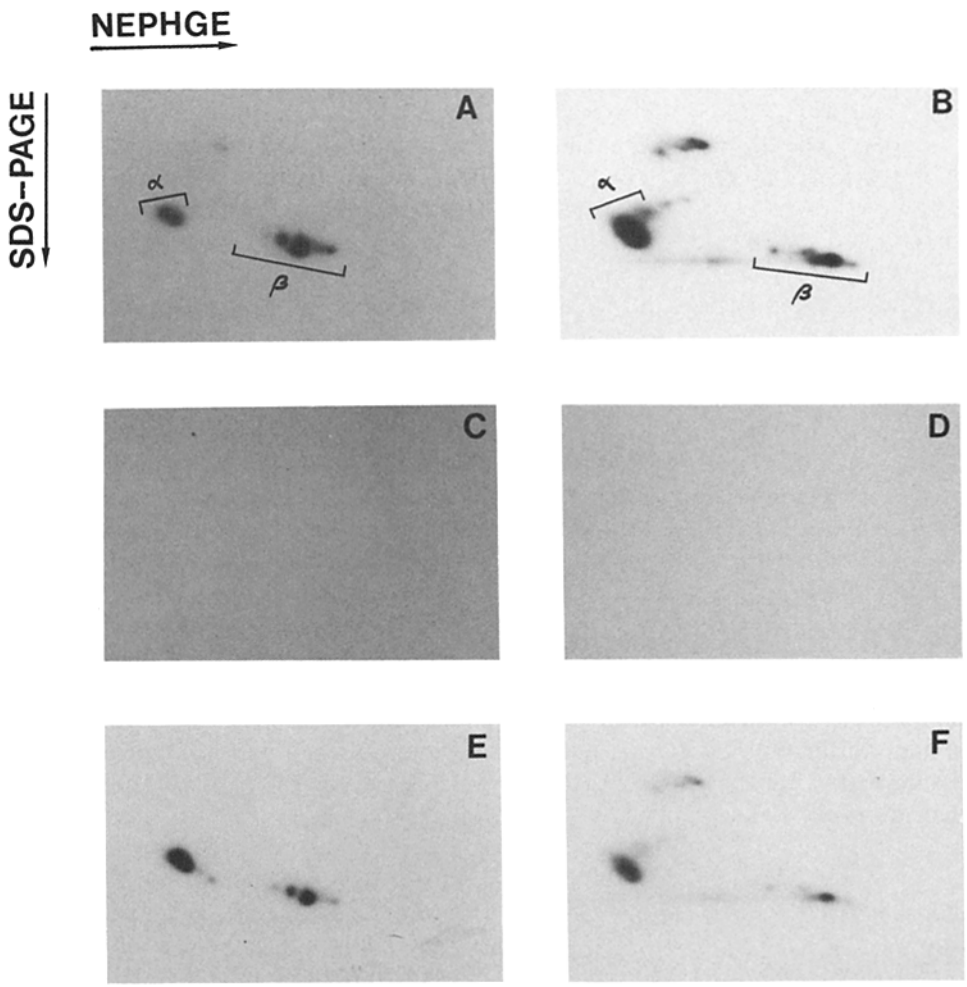

FIGURE 4. Two-dimensional PAGE analysis of specific immunoprecipitates obtained from extracts of ^{125}I cell surface–labeled Raji cells (*A* and *B*), RJ 2.2.5 cells (*C* and *D*), and RJ 2.2.5 × BALB/c spleen cell hybrid clone A2-2 (*E* and *F*). Cell extracts were immunoprecipitated with the DR-specific mAb D1-12 (*A*, *C*, and *E*), or with the DQ-specific mAb BT 3/4 (*B*, *D*, and *F*). α and β subunits of human class II molecules are indicated in brackets.

to mRNA preparations (Fig. 5, *center*) of the A2-2 and A2-6 hybrids (Fig. 5, *e* and *d*, respectively), whereas no specific hybridization was observed with either RJ 2.2.5 (*b*) or M12.4.1 (*c*) mRNA preparations. Although the dot blot hybridization assay used does not allow precise assessment of the amount of specific mRNA detected, the results obtained with the β-DQ probe suggest that, on a per cell basis, a lower amount of specific mRNA was present in the two hybrids when compared to the corresponding Raji cell hybridization pattern (*a*). This finding may help explaining why DQ molecules were reexpressed on the cell surface of the hybrids at a lower concentration as compared to the Raji cells (see Fig. 3 and 4).

Similar experiments were then performed by using the In chain–specific cDNA

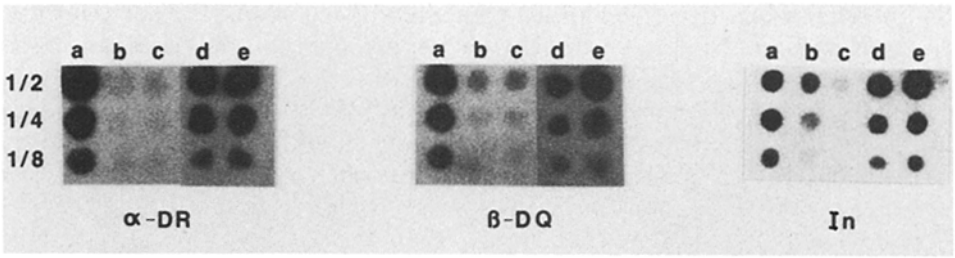

FIGURE 5. RNA dot blot hybridizations with: (left to right) DR α–, DQ β–, and In chain–specific probes. RNA was extracted according to White and Bancroft (37) from the following cell lines: Raji (a); RJ 2.2.5 (b); M12.4.1 (c); human × mouse hybrid clone A2-2 (d); human × mouse hybrid clone A2-6 (e). Numbers on the left side represent serial dilution of the samples. 10^7 cells were extracted in a volume of 100 μl; 20 μl of each sample were diluted and spotted on to nitrocellulose filters. DR α– and In chain–specific dot blots were exposed for 2 d. DQ β–specific dot blot was exposed for 5 d.

probe. We have previously shown (30) that In chain glycoproteins were synthesized in the RJ 2.2.5 Ia^- variant. This finding correlated with the presence of specific mRNA in the cytoplasm of RJ 2.2.5 cells, although at a reduced level compared to Raji cells (26).

The results presented in Fig. 5, *right* confirm that In-specific mRNA was reduced in RJ 2.2.5 cells (*b*) with respect to Raji cells (*a*). However, when the A2-2 and A2-6 hybrids were analyzed, a significant increase of specific mRNA was observed (*e* and *d*, respectively), attaining levels that were similar to those obtained in Raji cells, and suggesting that the transcription and/or the accumulation of this mRNA species was enhanced upon RJ 2.2.5 × mouse spleen cell hybridization.

Discussion

Previous studies (34) of our group have shown that the Ia^- phenotype of the B cell line RJ 2.2.5 could be suppressed at least to some extent after somatic cell hybridization with the mouse B lymphoma line M12.4.1. Because of the intrinsic characteristics of the hybrids (preferential segregation of human vs. mouse genome), it has been hard to assess whether reexpression of human Ia genes was regulated by a positive or negative *trans*-acting mechanism (see introduction and ref. 34). A prerequisite for such a study was, therefore, the availability of hybrids between RJ 2.2.5 and mouse cells capable of retaining human chromosomes while segregating the mouse counterpart. Such hybrids were obtained by using BALB/c spleen cells as fusion partners. The evidence that this set of hybrids preferentially retains human genetic material while losing mouse chromosomes resides in the fact that: (*a*) markers linked to human chromosome 6 (expression of class I antigens at the cell surface) were stably inherited; (*b*) mouse markers linked to chromosome 17 (such as class I and class II MHC antigens) were lost with time in culture not only at population level (see Fig. 1), but also from clones derived by limiting-dilution methods (Fig. 2, *B* and *D*), and more importantly, from subclones derived by micromanipulation from a highly purified clone displaying a mouse class I and class II–positive phenotype (groups B and D in Table III); and (*c*) the amount of mouse DNA in the hybrids, detected by

Southern blotting, decreased with time, while that of human DNA remained constant, and paralleled a reduction in number of mouse chromosomes as assessed by karyotype analysis (data not shown).

Studies at population level after selection of hybrid cells on the basis of both H-2K$^+$ and DR$^+$ phenotype revealed a progressive decrease, with time in culture, not only of mouse class I and class II antigen expression, but also of human class II expression (see Fig. 1). If we consider the expression of human and mouse class I genes as a measure of the stability of the respective genome, we can infer from Fig. 1 that loss of human class II antigen expression takes place with a similar rate as loss of mouse class I antigen expression. Such loss is not paralleled by loss of human chromosome 6, where class II genes are located, as indicated by the stability of expression of human class I markers.

Furthermore, studies of the clonal progeny of highly enriched DR$^+$, mouse MHC–positive populations, as well as on micromanipulated clones derived from a single progenitor, clearly revealed an instability of human class II expression mostly correlated with loss of expression of mouse markers. Again, even at clonal level, segregation of human chromosome 6 was never found.

From these results, we can conclude that loss of human class II expression in the hybrids is most probably correlated with loss of mouse rather than human genetic material. Within this context, we consider of importance the finding that, among the progeny of a cloned hybrid positive for the expression of class II antigens of both mouse and man, clones were found that lost, in a coordinate fashion, the expression of such antigens, still retaining the expression of murine class I antigens.

As a consequence of the above considerations, we suggest that class II gene expression is regulated by a diffusible factor, acting in *trans*, in a positive fashion, irrespective of species diversity. The origin of the Ia$^-$ phenotype in RJ 2.2.5 cells may thus be interpreted as a lesion in a locus coding for, or affecting the expression of such a factor.

Furthermore, the existence of cloned hybrids having lost human class II expression but not mouse class I expression (Fig. 2*C* and group C, Table III) or vice versa (Fig. 2*B* and group B, Table III) is a good indication that such a regulatory gene may not be carried by the mouse chromosome 17.

The developmental stages leading to expression of differentiated functions can be viewed as under the control of positive gene activation processes, and/or controlled by silencing developmental extinguishers. There are several well-characterized examples of differentiated functions that become extinct because of the possible presence of a developmental extinguisher in cells not committed to the expression of a differentiated phenotype (45). Examples of the reverse mechanism, that is, expression of a factor implementing gene expression only at appropriate developmental stages, are much less well documented. Regulation of expression of class II genes may be an example of a developmental activator at work.

In a previous study (34), we showed that somatic cell hybrids obtained by fusing RJ 2.2.5 cells with the mouse M12.4.1 B lymphoma cells expressed DR but not DQ antigens at the cell surface, and that this finding correlated with the appearance, in the cytoplasm of the hybrid cells, of substantial amounts of DR-

specific mRNA, and very low amounts (at the limit of detectability) of DQ-specific mRNA. In the present study, we have extended this observation by showing that mouse cells with a normal phenotype can restore the expression of human class II antigens upon hybridization with the Ia^- RJ 2.2.5 cells. RJ 2.2.5 × mouse spleen cell hybrids were characterized, as a population as well as at the clonal level, by expression of DR and DQ antigens, which did not differ, by biochemical criteria, from those expressed in Raji cells, the Ia^+ human B cell line from which the RJ 2.2.5 variant was generated. The expression of these two human Ia molecular subsets correlated with the presence of the respective specific mRNA in the cytoplasm of the hybrids. DQ still exhibited a somewhat reduced expression on the cell surface of the hybrids (see Results); the reasons for such behavior are unclear. This event may have depended on the hybrid nature of the cells, in which the residual mouse background may not allow the full expression of both class II gene families. However, other cases are known in which preferential expression of human DR vs. DQ has been reported (21, 24), suggesting that the expression of the two class II gene clusters could be regulated in a different manner.

The finding that, in the human class II–positive hybrids described in this study, the expression of In-specific mRNA was strongly increased indicates, as already suggested by analysis of RJ 2.2.5 (26), that not only the expression of human Ia genes, but also the expression of the In chain gene located on chromosome 5 (46) is controlled directly or indirectly by the mechanism(s) altered in RJ 2.2.5. This phenomenon acquires particular importance because the In chain gene product is specifically associated with Ia heterodimers during biosynthesis, and has been thought (27–29) to participate to the intracellular assembly and/or transport of the α-β subunits. Based on the possible involvement of the In chain glycoprotein in the biosynthesis of class II antigens, it is not surprising that the class II genes and the In chain gene may be coordinately regulated by a common control mechanism, or that class II gene products may themselves affect In chain gene regulation.

The presence of a seemingly sophisticated machinery determining the level of expression of class II genes suggests that gene regulation is an important parameter for the correct function of the immune system.

Summary

Raji, a human B lymphoma line, expresses high levels of major histocompatibility complex (MHC) class II antigens. Conversely, none of the detectable human Ia antigens is present in RJ 2.2.5, an immunoselected Raji variant. Clonal analysis, biochemical characterization, and nucleic acid hybridization studies of hybrids between mouse spleen cells and RJ 2.2.5 show that MHC class II gene expression is regulated in *trans* by a factor which, as judged by dominance studies, has the characteristics of an activator. Such a positive *trans* acting factor is expressed in mouse spleen cells, and is able to implement MHC class II gene expression across species boundaries. Expression of this factor in spleen cells strongly suggests that it plays a role in in vivo regulation of Ia expression. Additional data suggest that different subsets of class II genes such as DR and DQ may, in part, be regulated by different mechanisms. It has also been possible to show that the amount of In

chain–specific mRNA, present at reduced levels in RJ 2.2.5 cells compared to the parental Raji cells, drastically increased in human × mouse cells hybrids reexpressing human Ia antigens, suggesting that the In chain gene and the class II genes, although located on different chromosomes, are regulated in a concerted fashion, either directly through the same implementing factor, or indirectly through a cascade mechanism.

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