IMMUNOLOGICAL DISSECTION OF HUMAN Ia MOLECULES*

By Roberto Tosi,$ Nobuyuki Tanigaki,§ Dora Centi,‡ Giovanni Battista Ferrara, || and David Pressman§

From the Department of Immunology Research, Roswell Park Memorial Institute, Buffalo, New York 14263, Laboratory of Cell Biology, C.N.R., Via Romagnosi 18A, Roma, Italy and Immunohematological Research Center, AVIS Bergamo and Ospedale Generale Provinciale, Massa, Italy

Purely serological studies by means of complement-dependent cytotoxicity reactions against either chronic lymphatic leukemia cells (1) or B lymphocytes isolated from normal peripheral blood (2), has led to the identification of distinct human B-cell-specific alloantigens. Extensive population and family analysis through the cooperation program of the VII International Histocompatibility Workshop has defined eight individual specificities (3). These behave as products of alleles at a single locus and are also strongly correlated with the specificities of the HLA-D locus. Thus those allospecificities are designated as HLA-DR (HLA-D related) specificities.

However, many of the anti-B-cell antisera studied in the VII Workshop could not be included in the clusters corresponding to the eight defined DR specificities and some of them may recognize distinct non-DR specificities, as well as other still unidentified DR allelic products. Data obtained in the mouse system also show that Ia antigens which are specific to B lymphocytes are associated with at least three distinct I subregions (4) and different structural genes should code for the subunit(s) controlled by the H-2 region.

Independently from these serological studies, cell membrane glycoproteins which are expressed on B lymphocytes have recently been purified and characterized (5-7). They consist of two noncovalently linked polypeptide chains, i.e. a large subunit of 32,000–35,000 daltons and a small subunit of 25,000–28,000 daltons. They are not present on T lymphocytes. In this sense they are classified as B-cell-specific antigens and may well be the human equivalent of mouse Ia antigens. At least two loci, corresponding to the two basic subunits are involved in the synthesis of human Ia-like antigens. One of the two subunits is reportedly controlled by an HLA-unlinked locus (8).

* Supported in part by grant RO1 AI08899, awarded by the National Institute of Allergy and Infectious Diseases, Department of Health, Education, and Welfare by grants 5 R01 CA17276 and P 30 CA17609, awarded by the National Cancer Institute, Department of Health, Education, and Welfare, by NATO grant 1111 and by a grant from the U.S. Italy Cooperative Science Program.

∥ Laboratory of Cell Biology, C.N.R., Via Romagnosi 18A, Roma, Italy.

§ Department of Immunology Research, Roswell Park Memorial Institute, Buffalo, N.Y.; to whom reprint requests should be addressed, at: Roswell Park Memorial Institute, 666 Elm Street, Buffalo, N.Y. 14263.

|| Immunohematological Research Center, AVIS Bergamo and Ospedale Generale Provinciale, Massa, Italy.

Note on nomenclature: three terms are currently used, differing in their operational meaning, to define the same or related entities: (a) Ia-like antigens or molecules, (b) B-cell-specific antigens, and (c) HLA-DR antigens. We refer to Ia-like antigens as molecules possessing similar structures and tissue distribution as murine Ia antigens. The term B-cell-specific antigens will be used to generally indicate antigenic specificity present on B but not on T lymphocytes, irrespective of their representation on other cells. HLA-DR antigens (D locus related antigens) is a much more specific term indicating these alloantigenic determinants recognized by the clusters of antisera sorted out by the analysis of the data obtained during the VII Histocompatibility Workshop (3) and possessing a very high correlation with HLA-D locus specificities as defined by mixed lymphocyte culture reactions. Terms (a) and (b) will become equivalent only if all the alloantigenic specificities found on B but not on T lymphocytes will be shown to reside on the same molecular species and to have the same tissue distribution. Terms (b) and (c) can become synonyms only if the hypothesis of a single HLA-linked locus specifying all B-cell alloantigenic structures is true.
In the present work a fusion of the biochemical and immunogenetical approaches is attempted, with the aims of: (a) obtaining direct evidence of the association of a defined DR specificity as determined by cytotoxicity reactions with a specific molecular entity, and (b) testing whether the la molecule pool can be dissected by means of specifically reacting alloantisera into distinct subsets corresponding to different gene products.

The test system adopted has been the classical B-cell type cell line, Daudi, which has been used for studies of la-like antigens because it does not express HLA (A,B,C) molecules.

Materials and Methods

Cultured Cells. Cells of a human B-cell type cell line, Daudi, were cultivated in spinner culture or stationary bottle culture with RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. The cells for cytotoxicity tests were harvested at culture day 2 and used immediately. The cells used for isolation of human la molecules were harvested at culture day 2 or 3, washed with phosphate-buffered saline, packed at 600 g for 10 min and kept frozen at \(-70^\circ\text{C}\) until used.

The Daudi cells we used did not carry any HLA (A,B,C) allospecificity and did not produce \(\beta_2\)-microglobulin in accord with reports by others (9-11). Although, Ostberg et al. (12) have reported that the Daudi cells they used did contain HLA (A,B,C) antigens that can be detected by rabbit antiserum against HLA (A,B,C) antigens, we could not find such substances (7).

Preparations of \(^{125}\text{I}-\text{Labeled Human la Molecules.}\) Human la antigens partially purified from Renex 30 (Ruger Chemical Co., Irvington, N. J.)-solubilized membrane materials of Daudi cells by the method described previously (7) were radioiodinated and further purified by a series of procedures that involved enzymatic cleavage with papain (13).

Crude cell membranes were isolated by differential centrifugation from 10 g of Daudi cells (=5 \(\times\) 10\(^9\) cells) disrupted by nitrogen decompression (800 pound per square inch) (14). The crude membranes (wet weight 1.3 g) were homogenized with four parts of 2% Renex 30 in Tris-HCl buffer (iris 0.075 M, pH 7.8). After standing for 30 min at room temperature, the homogenate was centrifuged at 105,000 g for 60 min and the supernate was harvested. This solubilization process was repeated twice and the supernates were combined. The yield in A\(_{280}\) units was 172.

The Renex 30-solubilized membrane materials were then subjected to affinity chromatography on a Lens culinaris hemagglutinin (LcH)-coupled Sepharose column (LcH 4 mg/ml gel, bed vol 5 ml). The retarded fraction was eluted with a 2% mannoside solution. The recovery of A\(_{280}\) units in the retarded fraction was 4.8. The major portion of the retarded fraction was then fractionated by gel filtration on a Bio-Gel A1.5 M column (bed vol 100 ml). The human la activity, as assayed by the radioimmune inhibition assay (7), was found in fractions corresponding to about 200,000 daltons. The major fractions were pooled. The yield in A\(_{280}\) units was 0.41.

A small amount (0.014 A\(_{280}\) units) of the pool was labeled with radioactive iodine \(^{125}\text{I}\) by the chloramine-T method to give a specific radioactivity of about 20 mCi/A\(_{280}\) unit (15). The radiolabeled preparation was digested with papain in the presence of a carrier protein at 37°C for 30 min. The ratio of enzyme to substrate (1.0 ml of a 7.34 A\(_{280}\) units/ml carrier protein solution) was 1:40. The carrier protein used was the LcH unretarded fraction of Renex 30-solubilized membrane materials of Daudi cells. It was used after exposure to pH 2.3 for 30 min. This carrier did not contain any human la antigens, because la molecules are mostly removed on LcH affinity chromatography and are labile at acid pH (7).

Papain digestion split a substantial proportion of contaminants and also some of the la molecules leaving the remaining intact and thus increasing the purity of the preparation (13). After gel filtration of a Bio-Gel A1.5 M column (bed vol 50 ml), the separated modified la molecules (i.e. papain-split fractions) were further purified by LcH affinity chromatography as described previously (7).

Antisera. Heteroantisera used were the following: rabbit anti-\(\beta_2\)-microglobulin antiserum raised against a purified preparation of human \(\beta_2\)-microglobulin (16), rabbit anti-HLA (A,B,C) antiserum raised against a purified preparation of papain-solubilized HLA antigens (17), rabbit
IMMUNOLOGICAL DISSECTION OF HUMAN Ia MOLECULES

TABLE I

Synopsis of Relevant Antisera

| Antiserum          | Laboratory of origin | 7W number* | Nominal specificity$ | Specificity detected on Daudi Ia§ |
|--------------------|----------------------|------------|----------------------|----------------------------------|
| TH 21.3.75         | Thorsby              | DRw3 + 6   | DRw6                 |
| TH 19 Varen 75     | Thorsby              | DRw3 + 6   | DRw6                 |
| FD 8013 P1         | Engelfriet           | Unknown    | DRw6                 |
| 406                | Stastny              | 7W111      | DRw3 + 6             | DRw6 + weak DC-1                 |
| CLB 811.1          | Engelfriet           | 7W046      | DRw2 + 6             | DRw6 + DC-1                       |
| 19941/76           | Kissmeyer            | 7W066      | DRw2 + DRw1          | DC-1                              |
| Fordice 28.1.70    | Yunis                | Unknown    | Unknown              | DC-1                              |
| Cerny 7.12.72      | Yunis                | Unknown    | Unknown              | DC-1                              |
| CLB 833.2          | Engelfriet           | 7W007      | Unknown              | DC-1                              |
| Corazzola 418      | Jeannet              | DRw2 + DRw3| DC-1                              |
| Rettler 9.9.70     | Yunis                | 7W150      | Unknown              | DC-1                              |
| Fe 131/4, /6       | Ferrara              | Unknown    | Unknown              | DC-1                              |
| Fe 77/43           | Ferrara              | Unknown    | DC-1                              |
| Fe 96/11           | Ferrara              | Unknown    | DRw6 + DC-2          |
| Fe 67/15           | Ferrara              | Unknown    | DRw6 + DC-2 + x       |
| Fe 88/37           | Ferrara              | Unknown    | DRw6 + DC-2 + x       |
| Fe 75/39           | Ferrara              | Unknown    | DRw6 + DC-2 + x       |
| Moliner 567        | Jeannet              | DRw5       | DRw6 + DC-1 + x       |
| Betz               | Tanigaki (NIH bank)  | Unknown    | DRw6 + x             |
| T0-29-01           | Ceppellini (NIH bank)| Unknown    | Broad                |

* This is indicated for those antisera which were studied during the VII International Histocompatibility Workshop. More information on these sera can be obtained in the Workshop report (Histocompatibility testing 1977, in press).

$ Specificity either derived from the Workshop results or indicated by the laboratory of origin from the serological reactions in a local typed panel.

§ This column indicates the specificities that can be detected in these antisera when reacted with a purified Daudi Ia preparation. Each antiserum may obviously possess additional specificities.

anti-Fab fragments of normal human IgG, rabbit anti-B-cell membrane antiserum produced by immunization with crude membrane materials of a B-cell type cell line, RPMI-8235 (7), rabbit anti-B-cell glycoprotein antiserum raised against a crude preparation of membrane glycoproteins with LeH affinity obtained from a B-cell type cell line, B46M (7). In addition, rabbit anti-P23.30 antiserum that is specific to B-cell-specific antigens (5) was used. This was kindly provided to us by Dr. R. E. Humphreys, University of Massachusetts Medical School, Worcester, Mass.

Most of the alloantisera used in this work was obtained by planned immunization of blood donor volunteers selected according to their HLA phenotype. Details on this extensive immunization program have been previously reported (18, 19). Other reagents have been obtained by serum exchanges with other laboratories or from the NIH serum bank. All antisera were used without prior absorption with platelets. The antisera listed in Table I, after a preliminary screening of their cytotoxic and Ia binding activity, were selected for the immunological analysis of Daudi Ia molecules.

Direct Binding Test. Assay conditions have been previously described in detail (20). For the screening of the binding activity of antisera, equal portions of an $^{125}$I-labeled Ia preparation of
about 20,000 cpm were incubated with 10 μl of undiluted antiserum in the presence of 0.2% Renex 30 and 0.02% bovine serum albumin (BSA). After incubation for 16 h at 37°C, 70 μl of goat anti-human IgG serum was added. After further incubation for 30 min at room temperature, the immune precipitate was washed and the radioactivity was determined. A control with normal serum (in duplicate) was included in each experiment. Test with rabbit antisera was the same, except that 4 μl of antiserum, either undiluted or diluted in normal rabbit serum, were used, and 20 μl of goat anti-rabbit IgG was added as precipitating immune complex. Results obtained with alloantisera in different experiments were referred to the proportion of radioactivity bound by rabbit anti-B-cell membrane antiserum, by the following expression:

\[
\% \text{Ia binding} = \frac{\text{cpm bound by alloantiserum} - \text{cpm bound by normal human serum}}{\text{cpm bound rabbit anti-B cell membrane antiserum} - \text{cpm bound by normal rabbit serum}} \times 100.
\]

**Sequential Binding Test.** In experiments made either to determine the maximal proportion of Ia molecules which could be bound by a given antiserum or made to determine the relationship between the Ia molecules bound by the different antisera (cross-absorption experiments) the following procedure was used: the first binding step was carried out on 250,000 cpm of antigen with 50 μl of antiserum for 2 h at 37°C. After addition of 350 μl of goat anti-human IgG serum and further incubation at 37°C for 30 min, the precipitate was washed and assayed for radioactivity. The percentage of radioactivity bound was calculated with reference to the radioactivity bound by rabbit anti-B-cell membrane antiserum on the starting material.

The supernate recovered after the first absorption was combined with a second 50-μl portion of the same antiserum and incubated overnight at room temperature (second binding step). No precipitate formed, a result in agreement with our previous determination that no goat anti-human IgG antibody remained in the supernate. After another addition of 350 μl of goat anti-human IgG serum and further incubation (30 min, 37°C), the precipitate was washed and counted and the percentage of radioactivity bound calculated relative to the radioactivity bound by the rabbit anti-B-cell membrane antiserum on a portion of the supernate recovered after the first binding step. This percent of binding was expressed as fractional binding of the starting preparation; e.g., if the first binding was 19.4% of the total present and the second binding was 7.1% of the total present at that time, then the latter, relative to the starting preparation, is 0.71 × (100 - 19.4) = 5.7%, and the cumulative binding after two steps is 19.4 + 5.7 = 25.1. In cross-absorption experiments, portions of the supernate after the second binding step were allowed to react with different antisera under the same conditions.

**Cytotoxicity Test.** The ethidium bromide method (21) was used. Due to the peculiar sensitivity of cultured lymphoblastoid cells, the following precautions were adopted: (a) Daudi cells were harvested during early exponential growth, (b) complement was selected for its minimal cytotoxic effect with control sera coupled with a clear effect using antisera of different strength, (c) incubation times were shortened, as compared with those used for cytotoxicity test with normal peripheral blood lymphocytes, to decrease the background killing in the presence of complement.

The test was performed by adding 1 μl of cell suspension (1,000 cells) to 1 μl of undiluted antisera. After a 30-min incubation at room temperature, 1 μl of complement (undiluted rabbit serum) was added. After a further 30 min incubation, 3 μl of a 0.01% ethidium bromide solution was added and the dead cells were determined by their fluorescence. Live cells were determined as the difference between this number of dead cells and the total number of live and dead cells as determined under normal light. Percentages of dead cells were assigned on the basis of two independent readings. Scores were then assigned in the following way: score 1: 0-30% killing, 2: 30-50%, 3: 50-70%, and 4: 70-100%. The background killing controls (complement plus so called normal serum) could not be completely eliminated and amounted to 10-30%. Only scores 3 and 4 were thus considered as significant.

**Separation of the Large and Small Subunits of Human Ia Molecules.** 125I-Daudi Ia preparation was

---

2 **Abbreviations used in this paper:** BSA, bovine serum albumin; L/S, large subunit/small subunit ratio; SDS, sodium dodecyl sulfate.
Table II

Binding Characteristics of an $^{125}$I-Labeled Daudi Ia Preparation

| Antiserum                                      | Percent of radioactivity bound* |
|------------------------------------------------|---------------------------------|
| Rabbit anti-B-cell membrane (no. 5329)       | 72.2                            |
| Rabbit anti-B-cell glycoprotein (no. 7130)   | 65.4                            |
| Rabbit anti-P23-30                           | 63.8                            |
| Rabbit anti-HLA (A, B, C)                    | 0.9                             |
| Rabbit anti-#2-microglobulin                 | 1.0                             |
| Rabbit anti-Fab fragment                     | 1.7                             |
| Normal rabbit serum                          | 1.2                             |
| Alloantisera (TO-29-01)                      | 73.3                            |
| Normal human serum                           | 6.9                             |

* Equal portions of an $^{125}$I-labeled Daudi Ia preparation of about 20,000 cpm were incubated with 4 μl of each rabbit antiserum for 1 h at 37°C. The immune complex was precipitated by an excess of goat anti-rabbit IgG antiserum. The radioactivity in the precipitate was determined and expressed as the percent of the total added radioactivity. The amount of rabbit anti-HLA, anti-#2-microglobulin and anti-Fab fragment antisera was large enough to obtain the maximum level of binding in the reaction with the corresponding antigen preparation. In the case of TO-29-01 and its control, the data obtained by three-step cumulative binding test (Materials and Methods) are given.

Prepared as described above except that it was not subjected to papain digestion. 400 μl (9 × 10⁶ cpm) of the labeled preparation was incubated with 50 μl of a 10% sodium dodecyl sulfate (SDS) solution (no reducing agent was used) for 30 min at 56°C. The mixture was subjected to electrophoresis on a 12% gel (10 × 90 mm) containing 0.1% SDS (22). The gel was segmented at a 2 mm setting by an automatic gel divider (Gilson Medical Electronics, Inc., Middleton, Wis.). The separated portions were eluted by incubating each with a 0.5 ml of Tris-HCl buffer (Tris 0.075 M, pH 7.8) containing 0.2% Renex 30 and 0.02% BSA overnight at 4°C.

Results

The Purified Daudi Ia Preparation. Table II shows the binding properties of the radiolabeled Ia preparation which was utilized for the subsequent immunological analysis. The purity amounts to about 70%, as determined by two different rabbit anti-Ia antisera which could specifically recognize B-cell antigens in this preparation. Anti-P 23.30 antiserum gave approximately the same binding, indicating analogy with the Ia-like substance isolated by Humphreys et al. (5).

No significant binding was observed with antisera directed against other defined surface markers.

A broadly reacting alloantisera, TO-29-01, obtained from the NIH serum bank gave a binding similar (after subtraction of control binding) to that shown by rabbit anti-B-cell antisera. After three sequential steps, over 90% of the Ia molecules were bound by this antiserum, showing that most, if not all, Ia molecules carry alloantigenic determinants.

Analysis of the immunoprecipitates obtained with the different antisera by SDS-polyacrylamide gel electrophoresis showed a typical Ia pattern, with two spikes corresponding to a large (32,000 daltons) and a small (25,000 daltons) subunit of human Ia molecules (Fig. 1) as previously reported (7, 13).
Fig. 1. Analysis by SDS-polyacrylamide gel electrophoresis of Daudi Ia molecules bound to (a) rabbit anti-B-cell membrane antiserum and to (b) alloantiserum T0-29-01. A small amount (≈20,000 cpm) of 125I-labeled Daudi Ia preparation was allowed to react with 4 μl of rabbit anti-B-cell membrane antiserum or with 10 μl of T0-29-01. The soluble immune complex was precipitated with an excess of goat anti-rabbit IgG or goat anti-human IgG antiserum. The precipitate containing 14,000 cpm for rabbit anti-B-cell membrane and 10,000 cpm for T0-29-01 was treated with 50 μl of a 5% SDS solution (without reducing reagent) and subjected to electrophoresis on a 12% polyacrylamide gel (6 × 90 mm) containing 0.1% SDS (23). The gel was sliced into 40 segments from the cathode end by an automatic gel divider and each segment was assayed for radioactivity. The figures are plots of radioactivity against segment number. The curves are arranged to show that the patterns are essentially identical to each other with respect to number and position of the 125I-radioactivity peaks. The two major radioactivity peaks correspond to 32,000-dalton and 25,000-dalton subunits of Ia molecules as reported previously (7). The 125I-radioactivity peak preceding them may be an impurity in case of rabbit anti-B-cell membrane antiserum. In case of T0-29-01, it may be due to undissociated Ia molecules. In both cases, the last 125I-radioactivity peak is apparently due to a Ia fragment(s), because the control precipitate prepared with a normal rabbit serum or with a normal human serum did not give such a radioactivity peak (not shown).

Relationship between Complement-Dependent Cytotoxic Activity against Daudi Cells and Binding of Purified Daudi Ia Molecules by Alloantisera. A screening of 270 antisera from different laboratories was performed both by cytotoxicity assay and by direct binding assay. A general agreement was found between the two sets of data, as shown in Table III. Only three instances of positive cytotoxicity reactions were seen which were not coupled to significant Ia binding activity. On the other hand, at least seven antisera which were shown on repeated tests to be devoid of cytotoxic effect gave a significant Ia binding. The most evident example was serum Molimer which binds 77.8% of Ia molecules although being completely inactive in the cytotoxicity test.

All anti-DRw6 antisera tested gave strongly positive reactions on both tests. No positive results were given by the groups of antisera defining the other DR specificities. This agrees with the analysis performed on Daudi cells by different laboratories during the VII Histocompatibility Workshop (3), indicating that DRw6 is the only recognized DR specificity present on Daudi cells. Several antisera possessing anti-DC-1 activity (see below) gave either weak or negative cytotoxic reactions on repeated tests (Table III).

Immunological Analysis of Daudi Ia Molecules. The antisera which on the preliminary screening were found to possess significant binding activity were further studied to
define as closely as possible (with the limitations due to the generally low avidity of alloantisera) the proportion of Ia molecules that could be maximally bound. To this aim, each antiserum was titrated to find whether a plateau level of the maximum amount of Ia molecules bound could be obtained. Also, each antiserum was tested in sequential binding experiments with three subsequent additions of equal portions of antiserum. A cumulative binding proportion, as referred to the starting material, was thus determined.

A qualitative comparison between the Ia molecules bound by the different antisera was then made by reciprocal absorption experiments, i.e. by removing the molecules reacting with one antiserum and testing the remaining material with the other antisera.

Information on the proportion of molecules that each antiserum maximally binds was then combined with information on the specificity of each antiserum derived from the absorption data. A comprehensive picture of the alloantigenic heterogeneity of Daudi Ia molecules was thus obtained (Fig. 2). Three components (DRw6, DC-1, and DC-2) were defined with sufficient precision on the basis of different antisera sharing the same reactivity pattern. A portion of Ia molecules corresponding to about 25–30% of the total (shaded area, Fig. 2), remains undefined. Their immunological analysis must await the availability of other specific antisera. These molecules however do carry alloantigenic determinant, as shown by their reactivity with antiserum TO-29-01 (Table II), antiserum Fe 75/29 (Fig. 2), and antiserum Betz (Fig. 2).

Specificity DRw6. Because DRw6 was the only defined DR specificity detected on Daudi cells in the VII Workshop serological analysis, this was a logical starting point. Three antisera were available to us with nominal DRw6 specificity: two were Thorsby’s antisera (TH 21.3.75 and TH 19 Varen 75) and one was from Stastny (antiserum 406, corresponding to VII Workshop antiserum 111). In addition, an antiserum from Engelfriet, FD 8013P1 was found very similar to the Thorsby’s antisera and was classified as an anti-DRw6. All four antisera were strongly cytotoxic against Daudi cells. When titrated, it was apparent (Fig. 3) that none of the antisera reached a binding plateau at the maximum amount used (10 μl). A somewhat higher proportion of molecules could be bound by each antiserum by three sequential antiserum additions (Fig. 4). Antiserum 406 reacted weakly against an additional component (see below); the other three antisera, which were considered as representative of the DRw6 specificity gave an average cumulative binding of 30.2% (27.0, 29.2, 34.5).

Table III

| Cytotoxicity (% of cells killed) | Ia binding (% of radioactivity bound) |
|-----------------|-------------------------------------|
| >70             | 23 (8) 0 1                          |
| 50–70           | 7 (3) 2 2                           |
| 0–50            | 7 (4) 8 220                         |

*The antisera which possess anti-DC-1 specificity are indicated in parentheses.
FIG. 2. Immunological analysis of Daudi Ia molecules. Each antiserum is represented by a column of a length corresponding to the percentage of Ia molecules it can maximally bind, as determined by a three-step sequential binding assay (Materials and Methods). The position of each column reflects the results of cross-absorption experiments. The arrangement of the whole set of antisera is internally consistent. On the left hand side the designation of the identified subsets as well as their approximate contribution to the Daudi Ia pool is indicated.

FIG. 3. Titration of anti-DRw6 antisera. Plots of percent of Daudi Ia preparation bound against volume of various antiserum added. Equal portions of 125I-labeled Daudi Ia preparation containing about 20,000 cpm were incubated (24 h at 37°C) with different amounts of antiserum, either undiluted or diluted in normal human serum. Percentages of Ia binding were calculated after subtraction of the counts bound by 10 μl of normal serum and divided by the counts bound by rabbit anti-B-cell membrane antiserum, as indicated under Materials and Methods. Note that only antisera TH 21.3.75 and TH 19 Varen 75 appear to approach a plateau value at the largest amount of antiserum used.

FIG. 4. Proportion of Daudi Ia molecules cumulatively bound by four anti-DRw6 antisera used in sequence. Each column represents the percentage of Ia molecules bound at a given step plus the preceding step(s), calculated as described under Materials and Methods. Broken horizontal lines indicate the maximum binding observed in the direct titration of each antiserum, as shown in Fig. 3.
A further attempt to determine the total amount of DRw6-positive molecules was made by 10-fold increasing the antibody-antigen ratio as compared with the highest point in Fig. 3, i.e. by using 20 µl of antisera instead of 10 and 4,000 cpm of antigen instead of 20,000. Although under these rather extreme conditions unspecific binding may influence the results, the following binding percentages were obtained, TH 21.3.75: 39.8%, TH 19 Varen 75: 39.0%; FD 8013 Pl: 34.5%. It can be concluded that, with the limitation due to the low avidity of these antisera, a maximum of 40% of the Daudi Ia molecules, (but, more likely, a proportion lying between 30 and 35%) do carry the DRw6 determinant.

To establish whether the molecules bound by the three antisera were the same, reciprocal absorption experiments were performed. This kind of experiment is complicated if the maximum binding (plateau achieved) is not obtained in the determination with the first antisera. Even though two antisera may be reacting identically, absorption with one of them is only expected to reduce, not to eliminate the binding by the other. Table IV shows that absorption (two sequential steps) by any of the three antisera produced a strong reduction, as compared to controls, of binding by the other two. Additional proof that the three antisera recognize the same molecules is that their binding is reduced in a parallel fashion by absorption with other more complex antisera: CLB 811.1, Molimer, Betz, Fe 88/37, Fe 67/15, Fe 75/39 (data not shown).

**Specificity DC-1.** A group of 15 antisera was found in the preliminary screening possessing a similar binding capacity (≈15–20% binding). Out of these antisera four were chosen for more detailed study; of these two (19941/76 and Fordice) showed on titration a definite plateau of binding, while the others (Fe 96/11 and Fe 77/43) closely approached a plateau (data not shown). Data in Table V show that this is a homogeneous group of antisera, recognizing an alloantigenic determinant on Daudi Ia molecules. This new specificity was called DC-1. From the cumulative percent of binding obtained by the four antisera, the proportion of molecules carrying DC-1 can be estimated as 24.8% (average of 25.8, 24.8, 25.8, 22.8). Cross-absorption experiments show that DC-1 and DRw6 are carried by different molecules since absorption with antisera belonging to one group does not significantly reduce, in any of the combinations tested, the binding of antisera belonging to the other group. Most of the time, such absorption induced an enrichment of the molecules carrying the other determinant and thus an apparent increase of binding (Table VI).

Anti-DC-1 antibodies were detected in two other antisera, Molimer and CLB 811.1, by cross-absorption. Both antisera also contain anti-DRw6 antibodies (data not shown). Although Molimer is a broadly reacting antisera (77.8% of three-steps cumulative binding), CLB 811.1 appears to be a duo-specific antisera of DRw6 and DC-1 specificities because its cumulative binding (51.6%) fits the sum of DC-1 and DRw6-positive molecules (Fig. 2). Antiserum 406 also contains anti-DC-1 antibodies which can explain its higher binding, as compared with the other three anti-DRw6 sera (Fig. 3). Anti-DC-1 specificity was also found in some antisera studied in the VII Histocompatibility Workshop. For such antisera extensive serological data are available, because they have been tested with approximately 14,000 unrelated individuals. As shown in Table VII, all the antisera possess a highly significant correlation with DRw6 specificity.

**Specificity DC-2.** An additional determinant was identified on the basis of cross-
TABLE IV
Absorption Patterns by Anti-DRw6 Antisera*

| Absorbing antiserum‡ | Test antiserum | Ia binding | Reduction of Ia binding§ |
|----------------------|---------------|------------|--------------------------|
| TH 21.3.75 (25.1%)   | TH 21.3.75    | 12.6       | 52                       |
|                      | TH 19 Varen 75| 8.4        | 68                       |
|                      | FD 8013 P1    | 16.4       | 43                       |
| TH 19 Varen 75 (19.5%) | TH 19 Varen 75| 9.3        | 64                       |
|                      | TH 21.3.75    | 12.1       | 33                       |
|                      | FD 8013 P1    | 11.6       | 60                       |
| FD 8013 P1 (22.9%)   | FD 8013 P1    | 8.2        | 72                       |
|                      | TH 21.3.75    | 13.4       | 49                       |
|                      | TH 19 Varen 75| 10.9       | 58                       |
| Normal human serum   | TH 21.3.75    | 26.1       | —                        |
|                      | TH 19 Varen 75| 26.1       | —                        |
|                      | FD 8013 P1    | 26.9       | —                        |

Note that absorption by any of the three antisera produced a strong reduction, as compared to controls, of binding by the other two.
* An 125I-labeled Daudi Ia preparation of about 250,000 cpm was absorbed twice sequentially with 50 μl of a given antiserum. Equal portions of the supernate after the second absorption were allowed to react with the absorbing antiserum and with different antisera under the same conditions. The radioactivity bound was expressed as the percent of the total radioactivity added at the time. The control binding values (bottom of table) are those obtained with the labeled preparation absorbed twice, under the same conditions with normal human serum (Materials and Methods).
‡ The percentages of Ia molecules bound to the absorbing antiserum (two sequential absorption steps) are shown in parentheses.
§ This was calculated by dividing the experimental binding value (the preceding column) by the control binding value obtained with the labeled preparation absorbed with normal human serum.

absorption experiments, as a common specificity shared by three Ferrara's antisera: 88/37, 75/39, 67/15. None of these antisera is monospecific. All three contained anti-DRw6 antibody but did not contain anti-DC-1 antibody. They reacted with 73.1% (Fe 75/39), 65.5% (Fe 67/15), and 59.7% (Fe 88/37) of Ia molecules. Fe 75/39 included both Fe 67/15 and Fe 88/37, and Fe 67/15 included Fe 88/37. That these antisera recognize a common component, different from DRw6 was shown by the absorption pattern given by antiserum Betz. This antiserum contained anti-DRw6 antibody and others and bound 62.4% of Ia molecules in the three-step cumulative binding test. After a two-step absorption with Betz, 42.5% of Ia molecules remained. Out of these, 28.9, 23.3, and 27.8% (corresponding to 12.3, 9.9, and 11.8% as referred to the initial preparation) were bound by Fe 75/39, Fe 67/15, and Fe 88/37, respectively. The only way to accommodate at the same time the proportion of binding by each antiserum as well as the cross-absorption patterns is shown in Fig. 2.

3 The term monospecific has an operational meaning in this context: it refers to the property of an antiserum to recognize, in the Daudi Ia preparation, a seemingly single determinant, i.e. an antigen which cannot be dissected further by antisera recognizing a subspecificity (or split). These monospecific antisera may however contain antibodies against other antigenic determinants, not present in Daudi.
### Table V

Absorption Patterns by Anti-DC-I Antisera*

| Absorbing antisera $\dagger$ | Test antisera | Ia binding | Reduction of Ia binding $\ddagger$ |
|------------------------------|---------------|------------|------------------------------------|
| 19941/76 (25.6%)             | 19941/76      | 0.3        | 98                                 |
|                              | Fordice       | 0          | 100                                |
|                              | CLB 833.2     | 2.4        | 85                                 |
|                              | Corazzala 418 | 0.8        | 94                                 |
|                              | Cerny 7.12.72 | 0.5        | 97                                 |
|                              | Rettler 9.9.70| 0          | 100                                |
|                              | Fe 131/4      | 0.2        | 99                                 |
| Fordice (24.3%)              | Fordice       | 0.7        | 96                                 |
|                              | 19941/76      | 1.6        | 92                                 |
|                              | CLB 833.2     | 1.8        | 89                                 |
|                              | Corazzala 418 | 1.6        | 88                                 |
|                              | Cerny 7.12.72 | 1.0        | 93                                 |
|                              | Rettler 9.9.70| 0.9        | 94                                 |
|                              | Fe 131/4      | 1.4        | 91                                 |
| Fe 77/43 (21.7%)             | Fe 77/43      | 5.2        | 76                                 |
|                              | 19941/76      | 1.5        | 92                                 |
|                              | Fordice       | 1.2        | 93                                 |
|                              | CLB 833.2     | 1.7        | 89                                 |
|                              | Corazzala 418 | 1.3        | 90                                 |
|                              | Cerny 7.12.72 | 0.9        | 94                                 |
|                              | Rettler 9.9.70| 1.3        | 92                                 |
|                              | Fe 131/6      | 1.4        | 92                                 |
|                              | Fe 96/11      | 3.9        | 82                                 |
| Fe 96/11 (20.5%)             | Fe 96/11      | 2.9        | 86                                 |
|                              | 19941/76      | 0.5        | 97                                 |
|                              | Fordice       | 0.4        | 96                                 |
|                              | CLB 833.2     | 0.5        | 97                                 |
|                              | Corazzala 418 | 1.1        | 92                                 |
|                              | Cerny 7.12.72 | 0.3        | 98                                 |
|                              | Rettler 9.9.70| 0.6        | 96                                 |
|                              | Fe 131/6      | 0.2        | 99                                 |
|                              | Fe 77/43      | 5.3        | 75                                 |
| Normal human serum           | 19941/76      | 18.9       | —                                  |
|                              | Fordice       | 17.0       | —                                  |
|                              | CLB 833.2     | 15.9       | —                                  |
|                              | Corazzala 418 | 13.6       | —                                  |
|                              | Cerny 7.12.72 | 14.8       | —                                  |
|                              | Rettler 9.9.70| 15.5       | —                                  |
|                              | Fe 131/4      | 15.4       | —                                  |
|                              | Fe 131/6      | 17.3       | —                                  |
|                              | Fe 77/43      | 22.1       | —                                  |
|                              | Fe 96/11      | 21.4       | —                                  |

Note that absorption by any of the four antisera resulted in almost complete elimination, as compared to controls, of binding by all the test antisera.

* The experimental procedures are the same as described in Table IV.

$\dagger$ The percentages of Ia molecules bound to each antisera are shown in parentheses. Only one absorption step was required for the first two antisera to give essentially complete absorption.

$\ddagger$ This was calculated as described in Table IV.
### Table VI

**Evidence that DRw6 and DC-1 Determinants are on Different Molecules**

Absorption with Anti-DRw6 Antisera/Test with Anti-DC-1 Antisera

| Absorbing antiserum $|$ Test antiserum | Ia binding | Variation of Ia binding $|$ |
|-----------------|-----------------|-------------|-----------------------------|
| TH 21.3.75 (25.1%) | Fordice | 19.9 | +17 |
|                 | Fe 131/4 | 21.4 | +39 |
| TH 19 Varen 75 (19.5%) | Fordice | 19.3 | +14 |
|                 | Fe 131/4 | 19.6 | +27 |
| FD 8013 P1 (22.9%) | Fordice | 17.3 | +2 |
|                 | Fe 131/4 | 17.8 | +16 |
|                 | Fe 77/43 | 24.3 | +10 |
|                 | Fe 96/11 | 25.8 | +21 |
| Normal human serum | Fordice | 17.0 | — |
|                 | Fe 131/4 | 15.4 | — |
|                 | Fe 77/43 | 22.1 | — |
|                 | Fe 96/11 | 21.4 | — |

Absorption with anti-DC-1 antisera/test with anti-DRw6 antisera

| Absorbing antiserum $|$ Test antiserum | Ia binding | Variation of Ia binding $|$ |
|-----------------|-----------------|-------------|-----------------------------|
| Fe 77/43 (21.7%) | TH 21.3.75 | 25.4 | −3 |
|                 | FD 8013 P1 | 35.8 | +25 |
| Fe 96/11 (20.5%) | TH 21.3.75 | 27.5 | +5 |
|                 | FD 8013 P1 | 31.8 | +11 |
| 19941/76 (25.6%) | TH 19 Varen 75 | 32.7 | +25 |
| Fordice (24.3%) | TH 19 Varen 75 | 24.7 | −5 |
| Normal human serum | TH 21.3.75 | 26.1 | — |
|                 | TH 19 Varen 75 | 26.1 | — |
|                 | FD 8013 P1 | 28.6 | — |

Note that absorption with antisera belonging to one group does not significantly reduce, in any of the combinations tested, the binding by antisera belonging to the other group. Most of the time, such absorption induced an enrichment of the molecules carrying the other determinant and thus an apparent increase of binding as indicated by "+" sign.

* The experimental procedures are the same as described in Table IV.
$†$ The values in parentheses are the same as those given in Table IV and Table V.
§ This was calculated as described in Table IV.

i.e. assuming a component that we provisionally call DC-2, present on 10–12% of Ia molecules. DC-2-positive molecules can be operationally defined as those reacting with the three antisera 88/37, 75/39, and 67/15, but failing to react with the Betz antisera.

**Structural Characterization of DRw6 and DC-1 Molecules.** Because molecules carrying the DRw6 or the DC-1 determinant are bound by monospecific antisera, they could be separately analyzed for their subunit structure in SDS-polyacrylamide gel electrophoresis.
Correlation of Antisera Reacting against DC-1 with Defined DR Specificities

* From the VII Histocompatibility Workshop data for European Caucasoids (3). Only correlation coefficient ($r$) values $>0.20$ are reported.

‡ All the antisera listed are classified as anti-DC-1 antiserum, since their binding with an $^{125}$I-labeled Daudi Ia preparation was almost completely abolished by absorption with antiserum 19941/76 (7W066), a representative anti-DC-1 antiserum as shown in the present study.

![Fig. 5](image-url)

**Fig. 5.** Analysis by SDS-polyacrylamide gel electrophoresis of Daudi Ia molecules bound to (a) Fe131/4 (anti-DC-1), to (b) TH 19 Varen 75 (anti-DRw6), and to (c) CLB 811.1 (anti-DRw6 + DC-1). 10 μl of each antiserum was used. The other experimental conditions are the same as described in Fig. 1. Amounts of radioactivity in each immune precipitate were 3,200 cpm for Fe131/4, 4,100 cpm for TH 19 Varen 75, and 6,100 cpm for CLB 811.1. Note that the molecules bound to anti-DC-1 and to anti-DRw6 give essentially the same pattern in number and position of the radioactivity peaks. Also note that the radioactivity ratio of the large (32,000 daltons) subunit over the small (25,000 daltons) subunit is high for DRw6 (bound to TH 19 Varen 75) and low for DC-1 molecules (bound to Fe 131/4).

![Table VII](image-url)

**Table VII**

| Antiserum‡ | DRw1 | DRw2 | DRw3 | DRw4 | DRw5 | DRw6 | DRw7 | Ia8 |
|-----------|------|------|------|------|------|------|------|-----|
| 7W006     | 0.26 | -    | -    | -    | -    | 0.37 | -    | -   |
| 7W007     | 0.27 | -    | -    | -    | -    | 0.35 | -    | -   |
| 7W010     | 0.36 | -    | -    | -    | -    | 0.49 | -    | -   |
| 7W066     | -    | 0.44 | -    | -    | -    | 0.43 | -    | -   |
| 7W067     | -    | 0.27 | -    | -    | -    | 0.33 | -    | -   |
| 7W105     | -    | 0.52 | -    | -    | -    | 0.39 | -    | -   |
| 7W114     | -    | 0.33 | -    | -    | -    | 0.29 | -    | -   |
| 7W150     | -    | 0.41 | -    | -    | -    | 0.46 | -    | -   |
| 7W156     | -    | 0.45 | -    | -    | -    | 0.38 | -    | -   |
| 7W158     | -    | 0.36 | -    | -    | -    | 0.39 | -    | -   |

ALPHABETICAL LIST OF ANTI-DRw6 AND DC-1 ANTISERA

| DR specificities | r value |
|------------------|---------|
| DRw1             | 0.26    |
| DRw2             | 0.27    |
| DRw3             | 0.36    |
| DRw4             | 0.44    |
| DRw5             | 0.27    |
| DRw6             | 0.52    |
| DRw7             | 0.33    |
| Ia8              | 0.41    |

Phenotyping of Human Ia Molecules

| Antiserum‡ | DRw1 | DRw2 | DRw3 | DRw4 | DRw5 | DRw6 | DRw7 | Ia8 |
|------------|------|------|------|------|------|------|------|-----|
| 7W006      | 0.26 | -    | -    | -    | -    | 0.37 | -    | -   |
| 7W007      | 0.27 | -    | -    | -    | -    | 0.35 | -    | -   |
| 7W010      | 0.36 | -    | -    | -    | -    | 0.49 | -    | -   |
| 7W066      | -    | 0.44 | -    | -    | -    | 0.43 | -    | -   |
| 7W067      | -    | 0.27 | -    | -    | -    | 0.33 | -    | -   |
| 7W105      | -    | 0.52 | -    | -    | -    | 0.39 | -    | -   |
| 7W114      | -    | 0.33 | -    | -    | -    | 0.29 | -    | -   |
| 7W150      | -    | 0.41 | -    | -    | -    | 0.46 | -    | -   |
| 7W156      | -    | 0.45 | -    | -    | -    | 0.38 | -    | -   |
| 7W158      | -    | 0.36 | -    | -    | -    | 0.39 | -    | -   |
**Table VIII**

*Radioactivity Ratio between Large and Small Ia Subunits*

| Antiserum          | Specificity | L/S ratio | Mean  |
|--------------------|-------------|-----------|-------|
| TH 2.3.75          | DRw6        | 2.65      |       |
| TH 19 Varen 75     | DRw6        | 2.48      | 2.45  |
| FD 8013 P1         | DRw6        | 2.21      |       |
| 19941/76           | DC-1        | 1.62      |       |
| Fordice            | DC-1        | 1.78      | 1.74  |
| Fe 131/4           | DC-1        | 1.81      |       |
| CLB 811.1          | DRw6 + DC-1 | 2.06      |       |
| T0-29-01           | Broad       | 2.21      |       |
| Rabbit anti-B cell | Broad       | 2.11      |       |

*125I-labeled Daudi Ia molecules bound by a given antiserum were subjected to SDS-polyacrylamide gel electrophoresis and radioactivity carried by each of the large and small subunits was determined. Note that the ratios (L/S) are high for DRw6 molecules and low for DC-1 molecules. A mixture of DRw6 and DC-1 molecules shows an intermediate value.

determinant. However, when the ratio between the radioactivity counts in the first large subunit peak and in the second small subunit peak (L/S ratio) was calculated, a clear difference between DRw6 and DC-1 molecules was found (Table VIII). DRw6 molecules gave a high L/S ratio, while DC-1 molecules gave a low L/S ratio. As predicted, molecules bound by antiserum CLB 811.1 showed an intermediate ratio, while antisera which bind most Ia molecules showed a ratio somewhat lower than DRw6 antisera.

*Localization of Alloantigenic Specificities at the Subunit Level.* The two noncovalently linked subunits of which lamolecules are formed were separated by treatment with 1.0% SDS for 30 min at 56°C, followed by SDS-polyacrylamide gel electrophoresis and elution from the different gel fractions. The peak fractions corresponding to each of the large and small subunits were then allowed to react against rabbit anti-B-cell membrane antiserum and various alloantisera. Despite the strong denaturing conditions used, some antisera still bound to the small subunit. As shown in Table IX, relatively high binding was detected for at least one monospecific anti-DRw6 antiserum (TH 19 Varen 75) as well as with an anti-DC-1 antiserum (Fe 77/43). Comparatively high binding were given by more complex antisera, like Betz (DRw6 + x specificities), Molimer (DRw6 + DC-1 + x specificities) and CLB 811.1 (DRw6 + DC-1 specificities). Rabbit anti-B-cell membrane antiserum showed a 47.3% binding of the small subunit. No antisera gave a significant binding of the large subunit except rabbit anti-B-cell membrane serum which showed a 44.8% binding.

**Discussion**

The aim of this work has been to link information on B-cell alloantigens derived from extensive collection of population data based on serological typing with the information arising from the biochemical analysis of isolated cell membrane components.

From the data presented, the following conclusions can be drawn.

*Most B Lymphocyte-Specific Antigens of Daudi Cells are on Ia-Like Molecules.* This
TABLE IX

**Binding Activity of Isolated Ia Small Subunits by Alloantiseras**

| Antiserum       | Specificity     | Radioactivity bound$ | Binding§  |
|-----------------|-----------------|----------------------|-----------|
| TH 21.3.75      | DRw6            | 3,374                | 5.5 (2.2) |
| TH 19 Varen 75  | DRw6            | 10,332               | 16.0 (12.6)|
| FD 8013 P1      | DRw6            | 4,706                | 7.3 (3.9) |
| 406             | DRw6            | 2,459                | 3.8 (0.4) |
| Fordice         | DC-1            | 2,370                | 3.7 (0.3) |
| 19941/76        | DC-1            | 3,507                | 5.4 (2.1) |
| Fe 77/43        | DC-1            | 6,542                | 10.1 (6.7)|
| CLB 811.1       | DRw6 + DC-1     | 6,418                | 9.9 (6.5) |
| Molimer         | DRw6 + DC-1 + x | 3,402                | 8.3 (5.0) |
| Betz            | DRw6 + x        | 15,276               | 23.6 (20.2)|
| Fe 75/39        | DRw6 + DC-2 + x | 3,408                | 3.3 (1.9) |
| Fe 67/15        | DRw6 + DC-2 + x | 6,480                | 10.1 (6.7)|
| Fe 88/37        | DRw6 + DC-2 + x | 2,205                | 3.4 (0.0) |
| Normal          | —               | 2,183                | 3.4 (0.0) |

$ The values indicate radioactivity (counts/5 min) bound by 10 µl of serum, after subtraction of a background value (352 counts/5 min).

§ The values indicate the percentage of the radioactivity put in reaction (64,698 counts/5 min on the average). The figures in parentheses are the net percent binding, i.e. % binding with antiserum - % binding with normal serum.

* The large and small subunits of 125I-labeled Ia molecules were separated by SDS-polyacrylamide gel electrophoresis. Each subunit (about 13,000 cpm) was allowed to react against 10 µl of antiserum overnight at room temperature. Only the data for the small subunits are shown, since no antiserum tested gave a significant binding of the large subunit.

The Ia Binding Reaction Possesses a Sensitivity Comparable to That of the Complement-Dependent Cytotoxicity Reaction. The difference in sensitivity between the two tests can be better evaluated on the antisera whose specificity could be well defined, i.e. anti-DRw6 antisera and anti-DC-1 antisera. All four anti-DRw6 antisera were strongly and reproducibly cytotoxic and also gave very significant binding reactions. On the other hand, out of 15 anti-DC-1 antisera detected on the basis of their binding peculiarities, only eight gave strong cytotoxic reactions, three gave weak and poorly reproducible reactions, while the other four were completely negative.
The inconsistent cytotoxicity in this group of antisera appears to be not due to the class or the low avidity of antibodies but mostly due to the low concentration of the corresponding antigens, i.e. DC-1 molecules, on the surface membrane of Daudi cells (see below). Antiserum Molimer is also a remarkable example of a strongly binding antiserum (77.8% cumulative binding) and yet completely devoid of any cytotoxic effect on Daudi. This antiserum reacts with DRw6-positive molecules, besides other components (Fig. 2). In this case, the peculiarity is probably due to the strong predominance of a noncomplement fixing class of antibodies. Such examples have already been described (23, 24). It is also possible that this antiserum may possess a strong anti-complementary activity.

In general, it appears that anti-Ia alloantiserum have a higher affinity than anti-HLA (A,B,C) antisera (only a minor portion of this latter could be used in binding reactions [20]). Thus, the performance of Ia typing by radioimmunoassay seems now possible.

Only 30–35% of the Daudi Ia Molecules Appear to Carry the DRw6 Determinant. The determination of the proportion of the Daudi Ia molecules which carry DRw6 specificity was somewhat laborious, due to the rather poor definition of this antigen in the VII Workshop, to the lack of monospecific antisera and to their low affinity.

Three anti-DRw6 antisera behaved very similarly in binding and absorption experiments and the average of the maximum bindings obtained was assumed to express the proportion of the molecules which carry DRw6 specificity. However, the percentage of Ia molecules bound by these antisera only approached a plateau, without actually reaching it. The mean percentage bound, as obtained by the direct binding assay under standard conditions (overnight incubation at room temperature) with the maximum amount of antiserum used (10 μl), was 27.0% (26.1, 26.1, 28.9). This was increased to 30.2% (27.0, 29.2, 34.5) by performing three sequential binding reactions. By increasing the antibody-antigen ratio 10-fold as compared to the standard conditions, the average percent bound became 37.7% (39.8, 39.0, 34.5).

We feel that a figure of 30–35% for the proportion of the Ia molecules bound by anti-DRw6, while perhaps still being an underestimation, cannot be far from the actual contribution of the DRw6 allele to the Daudi Ia pool. A more definite determination will require either the availability of monospecific antiserum of a high affinity or the recognition of an allele to DRw6 expressed in Daudi.

A New B-Cell Alloantigenic Specificity (DC-1) Can Be Defined on Daudi Cells. This specificity could be precisely defined by the present immunochemical analysis because 15 different antisera were found to recognize the same subset of Daudi Ia molecules. It may appear strange that this antigen was not previously defined by serological analysis at the population level, because it is apparently a frequent specificity of antisera extensively studied in the VII Histocompatibility Workshop. A possible explanation lies in the fact that anti-DC-1 antisera usually appear to contain antibodies against other DR specificities, especially anti-DRw1 and DRw2 (see Table VII). The definition of DC-1 with the antisera by purely serological means would have then required extensive cellular absorption experiments. The availability of purified Ia preparation instead sorted out this specificity relatively easily. Thus, immunochemical approach, coupled with serological studies at the population level, can provide a powerful tool for the genetic analysis of this HLA region.

An additional specificity, DC-2 could be also defined by the immunochemical analysis, but only with complex, broadly reacting antisera. A search for monospecific
antisera against this component, as well as other antigenic components which may be present in Daudi cells is in progress.

*The DRw6 and the DC-1 Molecules are Probably Controlled by Separate, HLA-Linked Loci.* Two lines of evidence support this conclusion, genetic and biochemical, as follows: (a) genetic evidence—both DRw6 and DC-1 are controlled by HLA-linked genes. While there can be little doubt that DRw6 is controlled by the HLA region, the DC-1 specificity also appears to be HLA-linked because it is in linkage disequilibrium with DRw6. In fact, anti-DC-1 antisera studied during the VII Workshop show a high correlation coefficient (r) with DRw6 in the European caucasoid population (Table VII). If DC-1 and DRw6 were alleles, an opposite result, i.e. a negative correlation would be expected.

Because all antisera shown in Table VII may contain other antibodies, but did not certainly contain anti-DRw6 antibody, the only possible explanation of this correlation is linkage disequilibrium between DC-1 and DRw6, which in turn implies their control by two different closely linked loci. The presence of a third, though less well defined specificity, DC-2, strengthens this two-loci concept.

A possible explanation why a second segregating series has not been evidentiated by the VII Workshop analysis probably lies in the fact that the products of this second locus may be in general poorly immunogenic. This would make infrequent the occurrence of monospecific antisera, because they would be often contaminated by antibodies against more immunogenic DR antigens. We have previously provided evidence for a lower immunogenicity of non-DR, B-cell specificities (25). An analogy can be also drawn with the HLA-C locus, the discovery and analysis of which has been delayed because of a substantial difference in immunogenicity in respect to the A and B loci (26). Since the real proportion of DC-1-positive molecules in Daudi Ia is probably ≈10%, a further analogy can be established with C locus products, which also seems to be represented in lower amount than A and B loci products (27).

(b) Biochemical evidence: DRw6 and DC-1 molecules possess a similar two-subunit structure (Fig. 5). This is compatible either with allelism or with the control by closely linked, homologous loci, coding for the same types of subunits. However, in addition to the above genetic evidence, the fact that radioactivity ratio between the large and small subunits is different between DRw6 and DC-1 molecules suggests presence of differences in the basic structure and favors the linked loci possibility. The difference in radioactivity ratios is apparently due to a relative difference of iodination efficiency of the large and small subunits. It may well correspond to conformational differences in these two kinds of molecules. It may also reflect a difference in the number of tyrosine residues in either the large or the small subunit or in both of the subunits. If we assume that the large subunit is the same in both molecular species, then the data show that DC-1 molecules can incorporate, under the iodination conditions used, 40% more iodine into the small subunit than do DRw6 molecules. We have previously reported a similar finding in iodination of mouse H-2a antigens (H-2K^k and H-2D^d products); the large subunit of H-2K^k molecules contained more radioactivity than the small subunit, i.e. β_2-microglobulin while the reverse was true in case of H-2D^d molecules (28).

The two kinds of molecules also exhibited a marked (about five-fold) difference in papain sensitivity. This may recall the differences found among IgG subclasses (29).

Tanigaki, N., R. Tosi, K. Koyama, and D. Pressman. Purification and separation of subsets of human Ia molecules by papain digestion. Manuscript in preparation.
An analogy may be seen also with the murine system, where Ia genes belonging to different subregions appears to code for molecules which, though sharing a similar subunit structure, show distinctive structural features (30, 31).

**The Small Subunit Carries Alloantigenic Determinants Controlled by HLA-Linked Loci.** This conclusion stems from the binding data obtained in isolated subunits. Despite the extensive denaturation occurring during the isolation procedure and the possible loss of conformational determinants dependent upon subunit association, about 50% of both isolated large and small subunits could be bound by a rabbit anti-B-cell antiserum. Moreover, the small subunit showed a significant level of binding with some alloantisera (possibly endowed with higher affinity). At least one monospecific anti-DRw6 antiserum was among them. These results confirm our data reported previously (13). This is direct evidence that the small subunit is coded for by an HLA-linked gene. This finding agrees with the data of Schwartz et al. (32) showing that the small subunit carries la alloantigenic determinants in the guinea pig.

On the other hand, no definite conclusion can be drawn on the large subunit. In spite of its considerable binding activity with rabbit anti-B-cell antiserum, no significant binding was obtained with the alloantisera tested. This failure to react with alloantisera may indicate that the large subunit is a nonalloantigenic peptide which is common to all human Ia molecules, just like the β2-microglobulin in case of HLA (A,B,C) antigens. Alternatively, it may simply mean that its alloantigenic determinants are more sensitive to denaturation caused by the isolation procedure. There are data showing an homology between human Ia large and small subunits by tryptic peptide analysis (33). This may or may not be an indication of shared alloantigenic determinants.

In any case, the two subunits should be controlled by unlinked genes, if the rule for dimeric proteins composed of different subunits also holds in this case, as it does for lactic acid dehydrogenase, exoseaminidase A and B, hemoglobin and HLA (A,B,C) antigens. Our previous observation that the large and small subunits differ xenogenically one from another and have essentially no cross-reaction favors the concept of unlinked gene control (13). In fact, evidence from cellular hybridization studies indicates that the loci controlling the two subunits are not syntenic (8).

In conclusion, the following model is proposed: two HLA loci code for homologous, but distinct forms of the small Ia subunit. One of these is the DR locus; the second locus controls the DC-1 specificity in Daudi cells. The two loci are in linkage disequilibrium and thus they must be very close on the HLA region. The large subunit is probably controlled by an HLA-unlinked locus. This conclusion needs confirmation by genetic tests at the family and the population level.

**Summary**

The immunochemical analysis of Daudi Ia molecules by a variety of alloantisera led to the recognition of at least three molecular species carrying different antigenic determinants: DRw6, DC-1, and DC-2.

Genetic as well as structural evidence indicates that DRw6 and DC-1 molecules are controlled by separate, HLA-linked loci, rather than by alleles at the same locus. The alloantigenic determinants appear to be expressed on the small Ia subunit.

DC-1 and DC-2 determinants discussed had not been defined by serological analysis at the population level, but were demonstrated to be present by immunochemical analysis at the molecular level.
Most of the antisera used in this work have been obtained by exchange with other laboratories (Table I), whose collaboration is gratefully acknowledged.

We are indebted to the blood donors of the A.V.I.S. Association of Bergamo who volunteered for planned immunizations, thus giving a major contribution to research in this field.

Excellent technical assistance of Miss P. Overturf, Mr. A. J. Trott, Mr. R. Maturski, and Mrs. J. Shaver is deeply appreciated. We also thank Mrs. Bridget Winter, Mrs. Anna Konotopska, and Mr. Petro Ostapchuk.

Received for publication 20 June 1978.

References
1. Gossett, T., R. L. Walford, G. S. Smith, A. Robins, and G. B. Ferrara. 1975. The Merrit alloantigenic system of human lymphocytes. In Histocompatibility Testing. F. Kissmeyer-Nielsen, editor. Munksgaard, Copenhagen, Denmark. 682.
2. Wernet, P., R. J. Winchester, H. G. Kunkel, D. Wernet, M. Giphart, A. van Leeuwen, and J. J. van Rood. 1975. Serological detection and partial characterization of human MLC-determinants with special reference to B cell specificity. Transplant. Proc. 7(Suppl. 1):193.
3. Histocompatibility testing. 1977. Joint report. W. F. Bodmer, editor. Munksgaard, Copenhagen, Denmark. In press.
4. Murphy, D. B., K. Okumura, L. A. Herzenberg, L. A. Herzenberg, and H. O. McDevitt. 1976. Selective expression of separate I-region loci in functionally different lymphocyte subpopulations. Cold Spring Harbor Symp. Quant. Biol. 41:497.
5. Humphreys, R. E., J. M. McCune, L. Chess, H. C. Herrman, D. J. Malenka, D. L. Mann, P. Parham, S. F. Schlossman, and J. L. Strominger. 1976. Isolation and immunologic characterization of a human B-lymphocyte-specific cell surface antigen. J. Exp. Med. 144:98.
6. Snary, D., C. J. Barnstable, W. F. Bodmer, P. N. Goodfellow, and M. J. Crumpton. 1977. Cellular distribution, purification and molecular nature of human Ia antigens. Scand. J. Immunol. 6:439.
7. Koyama, K., K. Nakamuro, N. Tanigaki, and D. Pressman. 1977. Alloantigens of human lymphoid cell lines: 'human Ia-like antigens' alloantigenic activity and cell lines, organ and tissue distribution as determined by radioimmunoasssay. Immunology. 33:217.
8. Barustable, C. J., E. A. Jones, W. F. Bodmer, J. G. Bodmer, B. Arce-Gomez, D. Snary, and M. J. Crumpton. 1976. Genetics and serology of HL-A linked human Ia antigens. Cold Spring Harbor Symp. Quant. Biol. 41:443.
9. Ervin, P. E., and K. J. Nilsson. 1974. $\beta_2$-microglobulin production in vitro by human hematopoietic, mesenchymal and epithelial cells. J. Immunol. 112:137.
10. Fellous, M., F. Martchelewicz, M. Kamoun, and J. Dausset. 1975. The use of a lymphoid cell lines to define new B lymphocyte specificities, probably controlled by the MHC region. In Histocompatibility Testing. F. Kissmeyer-Nielsen, editor. Munksgaard, Copenhagen, Denmark. 708.
11. Jones, E. A., P. N. Goodfellow, J. G. Bodmer, and W. F. Bodmer. 1975. Serological identification of HLA-linked human 'Ia-type' antigens. Nature (Lond.). 256:650.
12. Ostberg, L., L. Rask, K. Nilsson, and P. A. Peterson. 1975. Independent expression of the two HL-A antigen polypeptide chains. Eur. J. Immunol. 5:462.
13. Tanigaki, N., K. Koyama, and D. Pressman. 1977. The component peptides of human Ia-like antigens. In Proteides of Biological Fluids, 25th Colloquium 1977. H. Peeters, editor. Pergamon Press, Oxford, England. 641.
14. Hunter, M. J., and S. L. Commerford. 1961. Pressure homogenization of mammalian tissues. Biochem. Biophys. Acta. 47:580.
15. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of $^{131}$I-labeled human growth hormone of high specific radioactivity. Biochem. J. 89:114.
16. Nakamuro, K., N. Tanigaki, V. P. Kreiter, and D. Pressman. 1974. Common antigenic
structures of HL-A antigens. IV. HL-A common portion fragment isolated from spent culture medium of human lymphoid cell lines. *Immunology.* 27:1127.

17. Nakamuro, K., N. Tanigaki, and D. Pressman. 1975. Common antigenic structures of HL-A antigens. VI. Common antigenic determinants located on the 33,000-dalton alloantigenic fragment portion of papain-solubilized HL-A molecules. *Immunology.* 29:1119.

18. Ferrara, G. B., R. Tosi, G. Azzolina, G. Carminati, and F. Kissmeyer-Nielsen. 1972. The production of anti-HL-A cytotoxic antisera through planned immunizations by intravenous injection of small aliquots of whole blood. *Tissue Antigens.* 2:359.

19. Ferrara, G. B., R. Tosi, A. Longo, A. Castellani, C. Viviani, and G. Carminati. 1978. A safe blood transfusion procedure for immunization against MH determinants in man. *Transplantation (Baltimore).* 26:150.

20. Ferrara, G. B., R. Tosi, A. Longo, A. Castellani, G. Carminati, and F. Kissmeyer-Nielsen. 1972. The production of anti-HL-A cytotoxic antisera through planned immunizations by intravenous injection of small aliquots of whole blood. *Transplantation.* 26:150.

21. Darke, C., and E. Gay. 1974. An evaluation of the ethidium bromide microcytotoxicity test. *J. Immunol. Methods.* 4:161.

22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680.

23. Colombani, J., M. Colombani, V. Lepage, and J. Dausset. 1973. Anticorps anti-HL-A ne fixant pas le complement. *C.R. Acad. Sci. (Paris).* 277:255.

24. Tosi, R. M., G. B. Ferrara, P. Antonelli, and A. Longo. 1975. Shift from HL-A antibodies active in C-dependent cytotoxicity to antibodies active only in ADCC after prolonged immunization from a single donor. In *Histocompatibility Testing.* F. Kissmeyer-Nielsen, editor. Munksgaard, Copenhagen, Denmark. 915.

25. Ferrara, G. V., A. Longo, A. Castellani, R. Tosi, O. Pugliese, G. Lombardi, P. G. Santi, N. Tanigaki, J. Minowada, G. Pellegris, M. T. Illeni, P. L. Tabacchi, F. Barboli, G. Carminati, C. Viviani, and M. Mennucci. 1977. Immunogenicity and complexity of B cell antigens. In *Histocompatibility Testing.* Joint report. W. F. Bodmer, editor. Munksgaard, Copenhagen, Denmark. In press.

26. Ferrara, G. V., R. Tosi, A. Longo, A. Castellani, C. Viviani, and G. Carminati. 1978. “Silent” alleles at the HL-A-C locus. *J. Immunol.* 121:731.

27. Snary, D., C. J. Barnstable, W. F. Bodmer and M. J. Crompton. 1977. Molecular structure of human histocompatibility antigens: the HLA-C series. *Eur. J. Immunol.* 8:580.

28. Natori, T., N. Tanigaki, D. Pressman, O. Henriksen, E. Appella, and L. W. Law. 1976. The component fragments obtained by acid dissociation of papain-solubilized H-2 molecules. *J. Immunogenetics.* 3:35.

29. Gergely, J., H. H. Fudenberg, and E. van Loghem. 1970. The papain susceptibility of IgG myeloma proteins of different heavy chain subclasses. *Immunchemistry.* 7:1.

30. Silver, J., W. A. Russell, B. L. Reis, and J. A. Frelinger. 1977. Chemical characterization of murine Ia alloantigens determined by the I-E/I-C subregions of the H-2 complex. *Proc. Natl. Acad. Sci. U. S. A.* 74:5131.

31. McMillan, M., J. M. Cecka, D. B. Murphy, H. O. McDevitt, and L. Hood. 1977. Structure of murine Ia antigens: partial NH2-terminal amino acid sequences of products of the I-E or I-C subregion. *Proc. Natl. Acad. Sci. U. S. A.* 74:5135.

32. Schwartz, B. D., A. M. Kask, and E. M. Shevach. 1976. The guinea pig MHC: functional significance and structural characterization. *Cold Spring Harbor Symp. Quant. Biol.* 41:397.

33. Springer, T. A., J. F. Kaufman, C. Terhorst, and J. L. Strominger. 1977. Purification and structural characterization of human HLA-linked B-cell antigens. *Nature (Lond.)* 268:213.