Proc. Nat. Acad. Sci. USA
Vol. 71, No. 5, pp. 2123–2127, May 1974

Immunological Identity of the Small Subunit of HL-A Antigens and β_2-Microglobulin and Its Turnover on the Cell Membrane

(immunoglobulins/lymphocytes/histocompatibility)

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Contributed by Jack L. Strominger, December 28, 1973

ABSTRACT **A number of immunological methods have been employed to show that the small subunit of HL-A antigens, isolated either after papain treatment or after solubilization with detergent, is identical to β_2-microglobulin, a protein previously isolated from human urine and shown to be homologous in structure to constant region domains of immunoglobulins. Moreover, quantitative data indicate virtually total identity between the small subunit of HL-A antigens and β_2-microglobulin. Studies of the turnover of labeled HL-A antigens from the lymphocyte surface indicate that the two subunits turn over at similar rates, although only the small subunit could be detected in the culture medium. The significance of these observations is discussed.**

HL-A antigens prepared from human lymphocytes by treatment with papain of either intact cells or membranes derived from these cells have been shown to contain two polypeptide chains of molecular weight approximately 34,000 and 12,000 by sodium dodecyl sulfate (SDS)-gel electrophoresis (1, 2). This finding was susceptible to two interpretations. Either the intact HL-A antigen complex on the cell surface contained the same two polypeptide chains or the two polypeptide chains arose by proteolysis due to papain. This question has been resolved by studies of HL-A antigens prepared after solubilization from membranes of human lymphocytes with detergents. Such preparations contained two polypeptide chains of molecular weight approximately 44,000 and 12,000; treatment of this material with papain resulted in cleavage of the 44,000-molecular-weight species to a species with a molecular weight of about 34,000 (3, 4). The 12,000-molecular-weight peptide is apparently not susceptible to papain. A peptide with HL-A "common" antigenic activity was also found as a component of human plasma and urine (5). Moreover, another peptide (molecular weight, 11,700) called β_2-microglobulin was first isolated from urine of patients with damaged kidneys (6) and has subsequently been found in normal human urine and serum (7, 8). Its amino-acid sequence possesses a substantial homology with those of constant regions of immunoglobulins. This finding has, for example, given rise to the speculation that β_2-microglobulin might be a "free immunoglobulin domain."

We have recently reported that the small subunit of HL-A antigen is identical to β_2-microglobulin in that β_2-microglobulin antisera bind all of the subunits of HL-A antigens in either detergent extracts or papain digests of human lymphocytes, and that the capacity of the antisera to bind HL-A antigens is specifically removed by absorption with pure β_2-microglobulin (9). Other experiments in two other laboratories have led to the same conclusion (10, 11). In the present paper further studies showing the immunological identity of the small subunit of HL-A antigens and β_2-microglobulin will be reported and quantitated, and studies of the shedding of the subunits of HL-A antigens from the surface of lymphocytes will be described.

Abbreviations: MW, molecular weight; SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

The materials and methods employed have been described in recent publications (1, 2, 4, 9).

RESULTS

Quantitation of β_2-Microglobulin in HL-A Antigen Preparations.

(*a*) Inhibition by HL-A antigen of binding of [^{125}I]β_2-microglobulin and antiserum.

Purified HL-A7,12 antigen prepared from RPMI 4265 lymphoblastoid cells following solubilization with papain (1) was tested for its ability to inhibit a radioimmunoassay for β_2-microglobulin using ^{125}I-labeled β_2-microglobulin purified from human urine and β_2-microglobulin antiserum. Inhibition curves obtained with the HL-A7,12 antigen indicated that an amount containing 8.75 ng of protein was required to reach 50% inhibition, while 2.06 ng of unlabeled purified β_2-microglobulin was needed to reach the same end point (Fig. 1). These data indicated that 23% of the protein of HL-A7, 12 antigen could be accounted for by β_2-microglobulin. Also, the fact that over 90% inhibition was obtained with the HL-A7, 12 antigen and that the shapes of the inhibition curves were similar suggested antigenic identity between the purified β_2-microglobulin and the material in the HL-A antigen.

(*b*) Inhibition of lysis of human lymphocytes by β_2-microglobulin antiserum.

β_2-Microglobulin antisera have been shown to bind to peripheral lymphocytes (7) and to lyse 20% of these lymphocytes (12). Using the ^{51}Cr cytotoxicity assay, it was found that essentially all normal peripheral blood lymphocytes could be lysed by rabbit antiserum to β_2-microglobulin in the presence of rabbit complement (Fig. 2*A*). By using various soluble HL-A antigens and purified β_2-microglobulin, it was possible to quantitate the amount of β_2-microglobulin contained in

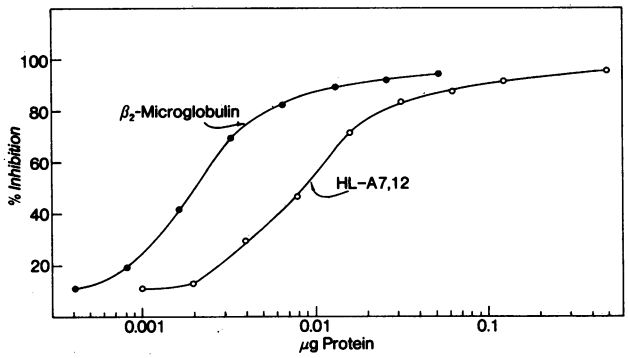

FIG. 1. Inhibition of precipitation of [^{125}I]β_2-microglobulin by rabbit antiserum to β_2-microglobulin with β_2-microglobulin purified from human urine or with HL-A7,12 antigen prepared after treatment of membranes of cultured human lymphoblastoid cells (RPMI 4265) with papain.

the HL-A antigen preparations by studying the capacity of these antigens to inhibit the cytotoxicity reaction (Fig. 2*B*). By this method it was estimated that for the papain-solubilized products 30% of the HL-A2 antigen preparation or 39% of the HL-A7,12 antigen preparation was composed of material crossreactive with β_2-microglobulin (Table 1). In a similar experiment with detergent-solubilized HL-A antigen preparations which were about 50% pure (3, 4) 12% of the inhibitory capacity of purified β_2-microglobulin was obtained, which, taking into account the larger molecular weight of the detergent-solubilized material and its degree of purity, closely correlates with the inhibition figures obtained with the papain-solubilized material. The data obtained with these two separate inhibition studies, although giving somewhat different values for the amount of β_2-microglobulin present in HL-A antigen preparations, clearly indicate that a large amount of β_2-microglobulin was present in these preparations (23–39%), considerably more than could be accounted for by contamination with an unrelated protein. This conclusion is strengthened by the fact that the preparative procedures for the two HL-A antigens isolated after papain digestion involved their elution at different salt concentrations in ion exchange chromatography (1) and that one of the steps in the purification of the detergent-solubilized material (4) was purification of glycoproteins by passage over a lectin column which would not bind a carbohydrate-free protein such as β_2-microglobulin unless it was bound to carbohydrate-containing molecules. The values obtained are in good agreement with the 35% by weight of the 12,000-MW polypeptide in papain-solubilized HL-A, antigen calculated on the ratio of [^{3}H]-aminoacids in the two polypeptides (1). The cytotoxicity assay measures only the subset of β_2-microglobulin antibodies directed at determinants exposed in the cell-bound form of β_2-microglobulin, yet approximately the same proportion of β_2-microglobulin was found in HL-A antigen by this assay as by the assay employing inhibition of [^{125}I]β_2-microglobulin precipitation assay. Therefore, most of the antigenic determinants of soluble β_2-microglobulin are also found in its cell-bound form.

Thus, quantitatively by two different methods, the small peptide of HL-A antigens was equally as effective as β_2-microglobulin in combining with β_2-microglobulin antisera. These data indicating virtually total cross reactivity appear to exclude the possibility that the 12,000-molecular-weight

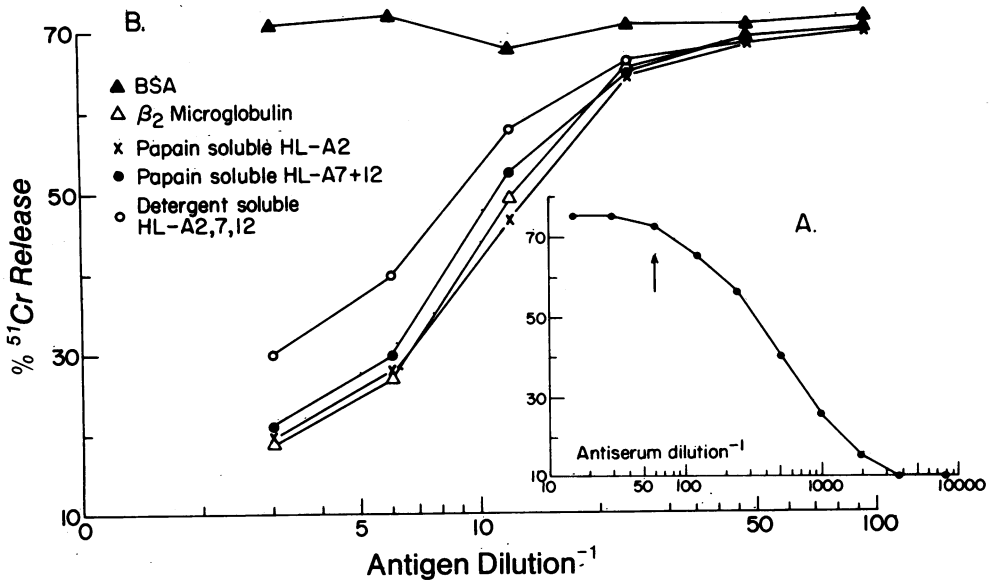

FIG. 2. Lysis of human peripheral lymphocytes by rabbit antiserum to β_2-microglobulin and inhibition of the lysis by HL-A and by β_2-microglobulin. (*A*) Lysis. The rabbit β_2-microglobulin antiserum was diluted serially 2-fold in 10 μl of dextrose–gelatin–Veronal–saline (DGV) plus 1:40 normal rabbit serum. Peripheral human lymphocytes (TS, HL-A.....) (5 μl containing 5 × 10^6 cells per ml, labeled with ^{51}Cr) were added and incubated 1/2 hr at 37°. Rabbit complement, diluted 1:4 with 0.14 M NaCl, 0.01 M Tris–glycine pH 8.3, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 0.01 mM ethylenediaminetetraacetate (EDTA), 100 μl, was added and the mixture incubated 45 min at 37°. Lysis was stopped by addition of 50 μl of 20 mM EDTA, 0.14 M NaCl, 0.01 M sodium phosphate, pH 6.8. After centrifugation at 1000 × *g* for 5 min, 50 μl of the supernatant solution was removed for ^{51}Cr counting. (*B*) Inhibition of lysis. Antigens were diluted in DGV plus 1:40 normal rabbit serum before assay. The amounts used are shown in Table 1. Detergent-soluble HL-A (4) which contained 0.34% Brij 99 (5 μl) was diluted with an equal volume of 30% bovine-serum albumin (BSA) before use. A sample containing BSA alone was also titrated and found to be completely noninhibitory. Antigens were diluted 2-fold serially through 1:40 β_2-microglobulin antiserum in DGV. After incubation for 1 hr at 37°, the assay was continued as described above.

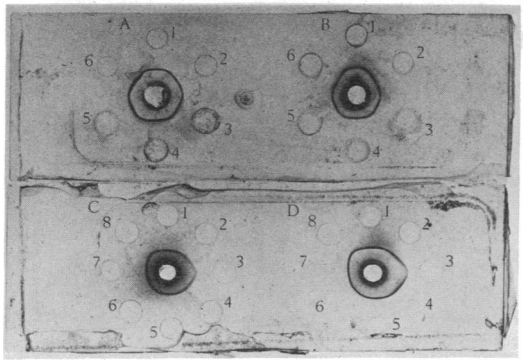

FIG. 3. Double diffusion of different HL-A preparations with antisera to β_2-microglobulin. (*A*) Center well, 12 μl of rabbit antiserum to β_2-microglobulin. Wells *1* and *4*, 0.5 μg of β_2-microglobulin; well *2*, 3 μg of papain-solubilized HL-A7; well *3*, 1.5 μg of same; well *5*, 1.5 μg of papain-solubilized HL-A2; well *6*, 3 μg of same. (*B*) Center well, 12 μl of turkey antiserum to β_2-microglobulin. Wells *1–6* as in *A*, above. (*C*) Center well, 12 μl of turkey antiserum to β_2-microglobulin. Wells *1* and *5*, 0.5 μg of β_2-microglobulin; well *2*, 1.5 μg of papain-solubilized HL-A7; well *3*, empty; well *4*, 4.5 μg of papain-treated detergent-solubilized HL-A2,7,12; well *6*, 6.5 μg of detergent-solubilized HL-A2,7,12; well *7*, 0.5 μg of papain-treated β_2-microglobulin; well *8*, 1.5 μg of papain-solubilized HL-A2. (*D*) Center well, 12 μl of rabbit antiserum to β_2-microglobulin; wells *1–8* as in *C* above. (The turkey antiserum was the generous gift of Mr. Harvey Faber, University of Wisconsin.)

subunit of HL-A antigen is a closely related polypeptide which cross reacts with β_2-microglobulin. It is noteworthy that, despite extensive homology, antisera to β_2-microglobulin do not cross react with immunoglobulins or vice versa.

Immunological Identity of the Reaction of Antisera to β_2-Microglobulin with HL-A Antigens and β_2-Microglobulin. The reactions of both rabbit and turkey antisera to β_2-microglobulin with HL-A antigens prepared after papain treatment or after detergent solubilization, and with β_2-microglobulin were examined by Ouchterlony double diffusion in agar. Lines of complete identity were obtained in all cases with no evidence of spurring (Fig. 3).

Identity of the Small Subunit of HL-A and β_2-Microglobulin by SDS and SDS–Urea Gel Electrophoresis and by Isoelectric Focusing. The various preparations of detergent- and papain-solubilized HL-A antigens and β_2-microglobulin were subjected to gel electrophoresis alone or together (Fig. 4*A* and *B*). In all cases the 12,000-molecular-weight peptides were found to be identical. On isoelectric focusing in 7.5% polyacrylamide gels containing 1% Ampholine, pH 4–6, β_2-microglobulin showed a single band having pI 5.2. Each of the HL-A preparations yielded a band in an identical position which on elution and SDS-gel electrophoresis had a molecular weight of

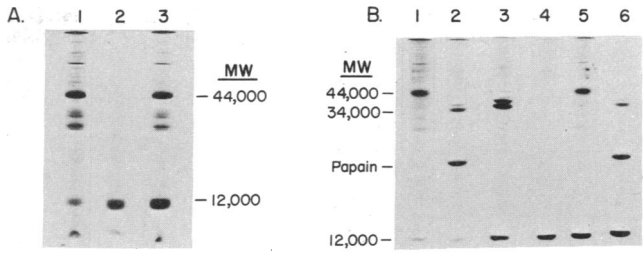

FIG. 4. Identity of β_2-microglobulin and the small peptide in various HL-A preparations. (*A*) SDS-gel electrophoresis (Laemmli SDS gels with 12% acrylamide). (*1*) 16 μg of detergent-soluble HL-A; (*2*) 216 μg of β_2-microglobulin; and (*3*) the two together. (*B*) SDS–6 M urea gel electrophoresis. (*1*) Detergent-soluble HL-A, 8 μg; (*2*) detergent-soluble HL-A, 8 μg, treated with 0.8 μg of papain; (*3*) HL-A solubilized from membranes with papain (1); (*4*) β_2-microglobulin; (*5*) sample *1* plus sample *4*; (*6*) sample *2* plus sample *4*.

12,000; the other bands in the HL-A preparations containing the large subunits were all more acidic (pI 4.2–5.1).

Kinetics of Turnover of the Two Subunits of HL-A Antigens from the Cell Membrane of Lymphocytes. To obtain information regarding whether the two HL-A antigens subunits are closely interrelated metabolically or not, experiments were performed to determine the fate of β_2-microglobulin and the larger subunit following lactoperoxidase-catalyzed radioiodination of the cell surface of lymphocytes. Three different preparations of lymphocytes were used, two from patients with chronic lymphatic leukemia and one of thoracic duct lymphocytes. Following labeling of the cell surface with ^{125}I, the cells were incubated for various times up to 6 hr at 37°. The supernatant fluids and a nonionic detergent lysate of the incubated cells were tested for the presence of radioiodinated β_2-microglobulin (the 12,000-MW subunit) and the 44,000-MW subunit by immunoprecipitation with an antiserum to β_2-microglobulin followed by SDS-gel electrophoresis of the reduced and alkylated immune precipitates. The data obtained from cells and supernatant fluid from lymphocytes of a patient with chronic lymphatic leukemia are shown in Fig. 5. The rates of disappearance of the two polypeptide chains from the cell surface were quite similar in a given experiment; however, the rate of disappearance markedly differed from one cell type to another. For instance, cells from the patient shown in Fig. 5 had only lost about 30% of both β_2-microglobulin and the 44,000-MW subunit after 6 hr, whereas lymphocytes from another patient with chronic lymphatic leukemia had a half-time of 90 and 120 min for β_2-microglobulin and the 44,000 MW subunit, respectively. The thoracic duct lymphocytes had a half-time of 240–270 min for the two subunits. Analysis of the supernatant fluids for the 44,000-MW and 12,000-MW components revealed only the 12,000-MW subunit to be pres-

TABLE 1. *Inhibition of immune cytolysis by HL-A antigens and β_2-microglobulin*

Antigen	Conc. (mg/ml)	Dilution before assay	Titer	Absolute titer	Titer mg/ml	% microglobulin
Papain-solubilized HL-A2	0.20	21	9.4	197	990	30
Papain-solubilized HL-A7,12	0.13	21	8.2	172	1300	39
Detergent-soluble HL-A2,7,12	0.33	22	6.0	132	400	12
β_2-Microglobulin	2.7	1000	9.0	9000	3300	(100)

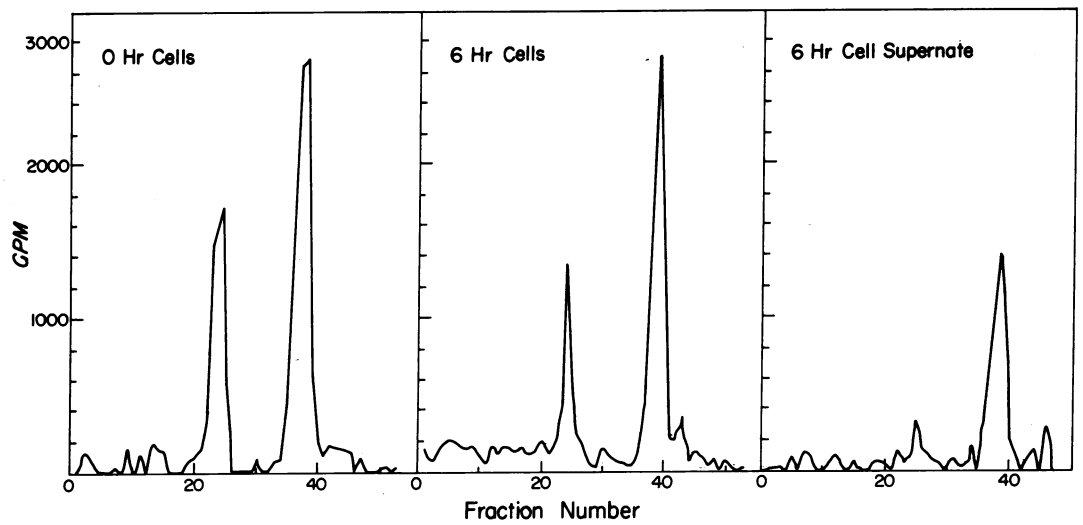

FIG. 5. Turnover of [^{125}I]HL-A antigen on lymphocytes from a patient with chronic lymphatic leukemia and appearance of the small subunit (β_2-microglobulin) in the supernatant fluid. Gels were labelled by the lactoperoxidase method as employed previously (9). For description of experiment, see *text*.

ent in significant amounts. At least 50% of the β_2-microglobulin which left the surface of the cell was recoverable from the supernatant fluid; none of the 44,000-MW component which left the surface was recovered. These experiments suggest that two polypeptide chains of HL-A antigen are metabolized at similar rates; however, different cell sources seem to have quite different metabolic turnover rates. A large portion of the turnover of the β_2-microglobulin component appears to be via shedding into the tissue culture medium, whereas the 44,000-MW component, although turning over at a similar rate to the β_2-microglobulin, does not appear to be shed into the culture medium, or if it is, it is shed unbound to β_2-microglobulin and, therefore, not precipitable by the β_2-microglobulin antiserum. An alternative mechanism of turnover of the 44,000-MW subunit would be intracellular metabolism or exchange with an intracellular pool. These experiments are compatible with studies which have resulted in the identification of the 12,000-MW subunit of HL-A antigen, unassociated with the 44,000-MW subunit, in human serum and urine (5–8).

DISCUSSION

These findings together with those presented earlier (9–11) then lead to the conclusion that the small subunits of HL-A antigens and β_2-microglobulin are identical, or so closely related that they cannot be distinguished by immunological means. β_2-Microglobulin is a protein of molecular weight 11,700 which is found in human urine and has already been sequenced (6–8, 13). It is moderately homologous in structure to the constant region domains of the heavy chain of IgG, showing the greatest homology with the C_{H} III domain. The association of β_2-microglobulin with HL-A antigens leads to the speculation that the large subunits of HL-A antigens may also be structurally related to the large subunits of immunoglobulins. Both are glycoproteins, HL-A having a molecular weight of 44,000 (3, 4) and the heavy chains of IgG, 50,000. Also, it has been reported that a rabbit antiserum against the Fc fragment of human IgG (although not antisera against the IgG itself or against the Fab fragment) lyses human lymphocytes and appears to be inhibited by purified HL-A (14); this reaction could be directed to either the small subunit or large subunit of HL-A or both.

Moreover, it is noteworthy in this connection that although the two chains of IgG and most other immunoglobulins are linked by disulfide bonds, a class of immunoglobulins called IgA_2 in which the two polypeptides are linked by noncovalent forces has been identified (15). In addition both the K and λ chains of IgG have been shown to interact with the heavy chains by noncovalent forces as well as by disulfide bridges (16). The two subunits of HL-A antigens are not linked by disulfide bonds (2), and in this respect would be similar to the class of immunoglobulin represented by IgA_2.

Two major pieces of evidence have been presented to indicate that β_2-microglobulin is a subunit of the HL-A antigen complex. First, when HL-A antigen was isolated by procedures which should have eliminated any significant contamination of low-molecular-weight material (<20,000, ref. 1) or of material that had no significant carbohydrate moiety (4), β_2-microglobulin—a low-molecular-weight carbohydrate-free protein—was found to be present in amounts approximating 25–35% of the total weight of the isolated HL-A antigens. Additional compelling experiments concerning the nature of the β_2-microglobulin–HL-A relationship were performed on internally labeled cells (1, 9). The ability of the supernatant solution of papain-treated cells to combine with HL-A antiserum of suitable specificity was completely abrogated by previous treatment of the supernatant with β_2-microglobulin antiserum and removal of the resulting β_2-microglobulin–anti-β_2-microglobulin complexes (9). This experiment clearly indicates that the large glycopeptides of HL-A antigens are in intimate association with β_2-microglobulin. The question may, however, be raised as to whether this association is fortuitous and occurred after solubilization, or whether it reflects an association which actually occurs on the cell surface. The latter seems more likely, since the two subunits require rather drastic conditions for separation, are found together whether the HL-A is solubilized by detergent or by proteolysis with papain, and turn over at similar rates.‡

‡ Recent data indicating that the two subunits cap together provide further support for their association on the membrane (21).

Since nothing is known of the function of either β_2-microglobulin or HL-A on the cell membrane, it is difficult to speculate what the association between these two proteins signifies. However, because of the homology between β_2-microglobulin and immunoglobulin polypeptide chains, along with the association of histocompatibility genes with genes for immune responsiveness, a discussion of the possible role of the β_2-microglobulin–HL-A complex in the immune response appears warranted. In the mouse H-2 histocompatibility system (17), and more recently in the HL-A system (18), it has been clearly demonstrated that immune responsiveness to certain antigens is controlled by a gene, *Ir*, closely linked to the major histocompatibility loci. That it is not identical with the known histocompatibility loci has been clearly shown by recombinant studies in which the *Ir* gene effect and the D and K end specificities of H-2 antigens can be separated (19). The nature of the *Ir* gene product is totally unknown, but since it influences the immune response, apparently by governing the T (thymus-dependent) helper cell effect, the possibility exists that the *Ir* gene product is an antigen receptor on T cells (17). The fact that H-2 antigen itself, although closely linked to the *Ir* gene product, is not identical with it, would appear to make it somewhat difficult to postulate a role of the β_2-microglobulin in association with a histocompatibility antigen as representing the T cell antigen receptor. However, there is suggestive evidence that the *Ir* gene product may itself be the product of a histocompatibility-like gene, since strong cytotoxic alloantisera are raised against it (19, 20). It is possible, therefore, that besides the known histocompatibility antigens, other products of the "immune responsiveness" genetic region exist (of which the *Ir* gene product is one) and β_2-microglobulin in association with this product acts as an antigen receptor on T cells.

A second possibility is that the *Ir* gene codes for a V (variable)-region-like peptide chain with antigen-binding capability and that, as in Ig genetics, the product of this gene in covalent linkage with a C (constant) region makes up a single polypeptide chain. In this case, it would be postulated that the C region is represented by the H-2 or HL-A antigen. It should be pointed out that structural studies on the H-2 antigen have not revealed any significant degree of heterogeneity as would be expected if a V region were present. However, methodology designed to reveal subtle degrees of heterogeneity has not been utilized yet in the characterization of histocompatibility antigens. Techniques such as peptide mapping and amino-acid composition analysis have revealed few differences among different H-2 and HL-A antigens; however, these methods are not sensitive enough to detect a great degree of heterogeneity in a limited portion(s) of a given molecule. It is even possible that in the membrane a V and C region could be associated by noncovalent bonds.

With respect to β_2-microglobulin heterogeneity, two laboratories have reported sequence data which indicate that at least for the first 45 residues there is no significant degree of heterogeneity present (7, 8, 13). It is likely, therefore, that, if β_2-microglobulin is part of an antigen receptor, its role would be predominantly one of modulation of an antigen combining site existing on another polypeptide chain.

These studies were supported by research grants from the U.S. Public Health Service (AI-09576 and AI-09578)

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