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Transplantation Antigens

Solubilized antigens provide chemical markers of biologic individuality.

Barry D. Kahan and Ralph A. Reisfeld

The fate of tissue grafts (histocompatibility) depends upon the genetic relation of the donor to the host. The historic experiments of Loeb (*1*), Little (*2*), and Snell (*3*) demonstrated that grafts exchanged between members of the same inbred strain (isografts) survive permanently, while grafts exchanged between members of two different strains (allografts) are promptly rejected. There are at least 15 histocompatibility loci controlling transplantation in mice (*4*), eight in rats (*5*), four to six in guinea pigs (*6*), and four in Syrian hamsters (*7*). The strength of the individual genetic differences is believed to be related to the speed of graft destruction when donor and host differ solely at that locus. If the graft survives less than 14 days, donor and host are defined to be incompatible at a strong transplantation locus. On the other hand, graft rejection due to weak genetic differences does not occur until 16 to 200 days after transplantation (*8*). In each species which has been carefully investigated, there is a single strong histocompatibility locus controlling the rapid rejection of allografts: the H-2 locus of mice (*3*), the HL-A locus of man (*9*), the Ag-B (H-1) locus of rats (*10*), and the B locus of chickens (*11*).

The mechanism of the rejection phenomenon was not immediately apparent. Loeb (*1*) postulated that grafts release foreign substances which initiate primarily local, cellular reactions leading to rejection. Twelve years later Gibson and Medawar (*12*) noted that skin allografts applied to a patient who had rejected previous grafts from the same donor were destroyed in accelerated fashion—the second-set phenomenon. In a series of elegant experiments in outbred rabbits Medawar (*13*) demonstrated that the second-set phenomenon was specific for the donor of the first (sensitizing) graft, and that the resistance induced by the initial transplant was systemic, that is, grafts applied onto any site were destroyed in accelerated fashion. He concluded that local events did not determine the fate of the graft and proposed the immunologic hypothesis of rejection: after transplantation grafts release substances (antigens) which induce an immune response against themselves. Presumably it is the antigenic product (or products) of the strong transplantation locus or, in some instances, of multiple weak loci operating in concert (*14*), which are the prime movers in the rejection phenomenon and thus of the greatest biologic interest. Serologic studies have suggested that the gene product or products possess several antigenic specificities, and genetic studies suggest that these are determined by a single chromosomal region (*9*, *15*).

The gene product of this chromosomal region appears to be essential for cell function. Determinants of transplantation antigens can be demonstrated on all cells and can be detected on cells perpetuated in tissue culture (*16*). In that allografting represents a situation not known to occur in nature, it would be expected that survival pressure would have discarded these components unless they played a significant role in cell structure or function. It has been postulated that these substances mediate either transport (*17*) or, more probably, cell contact and recognition phenomena (*18*). Presumably, in the course of performing their natural function, these potentially antigenic substances are recognized as foreign by the host's immune system and become the target of his response.

Assay Systems

The products of the strong transplantation loci appear to have three biologic actions which presumably relate to histocompatibility: (i) the induction of allograft immunity, (ii) the evocation of humoral alloantibodies, and (iii) the elicitation of specific cutaneous hypersensitivity reactions. According to rigorous criteria, a substance must affect the fate of donor-specific grafts, either by hastening their

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destruction (the second-set phenomenon) or by prolonging their survival, to be a "transplantation antigen." The second-set method is relatively sensitive: 1.3×10^{-10} mole of water-soluble, purified guinea pig transplantation antigen suffices to hasten the rejection of allogeneic skin grafts (*19*). However, the test is cumbersome and requires an interval of at least 2 weeks between immunization and final histologic analysis, and it is subject to quantitation only with difficulty (*20*). The opposite end of the spectrum of antigenic activity, the prolongation of graft survival, has been obtained by prior treatment of the prospective host with high doses of subcellular antigen prepared by homogenization (*21*) or by sonication (*22*) in conjunction with immunosuppressive agents. In addition to this zone of large antigen doses (overloading, paralyzing), Mitchison (*23*) has found a zone of low-dose tolerance wherein administration of antigen in amounts insufficient to sensitize hosts may result in the development of specific immune tolerance (*24*). Although the tolerance system appears to be inconvenient and time-consuming, it carries a unique biologic significance. Whereas the induction of immunity requires only a single foreign antigenic determinant in the extract, immunologic tolerance requires that all of the disparate antigenic determinants be present.

Because the methods employing tissue grafting are cumbersome, rapid techniques based upon other immunological properties of the products of the histocompatibility loci have been developed. Subcellular extracts which are active only in these systems, and not in allografting assays, are best denoted as "histocompatibility substances" in order to distinguish them from materials with proven effects on graft survival. Histocompatibility substances are assumed to represent the antigenic determinants separated from the carrier which is essential for biologic activity.

Four types of delayed hypersensitivity reactions have been developed for antigenic analysis: the direct reaction, the transfer reaction, the irradiated hamster test, and the blast transformation phenomenon (*25*). These systems reflect the interaction between immunocompetent sensitized cells and transplantation antigen independent of the end-stage process of graft destruction. Since first-set allograft rejection is apparently mediated by delayed-type hypersensitivity, these systems reflect the performance of antigens in reactions involving the cellular effector mechanism of transplantation immunity. Furthermore, since the sensitized cells are obtained from animals which have just rejected allografts, the ability of extracts to elicit these hypersensitivity responses implies that, in the development of transplantation immunity, hosts develop specific sensitivity toward these materials.

After prior sensitization with allogeneic tissue grafts, guinea pigs (*25, 26*), mice (*27*), humans (*28*), and hamsters (*29*) develop delayed (tuberculin type) cutaneous hypersensitivity responses to intradermal challenge with donor-type cells, crude extracts, or purified antigens; this is the direct reaction. Specific immediate hypersensitivity reactions (Arthus reactions) can be elicited by intradermal challenge of human donors of mono- or oligospecific antiserums with antigenic extracts from human tissues (*30*).

Brent *et al.* (*26*) have demonstrated that the inoculation of sensitized guinea pig lymphocytes into the skin of the graft donor produces a violent, protracted delayed-type cutaneous hypersensitivity phenomenon; this is the transfer reaction. This phenomenon represents the response of the transferred cells to the transplantation antigens either residing in or brought to the test sites by host leukocytes. Third-party transfer reactions have been obtained by intradermal inoculation of an admixture of sensitized leukocytes and allogeneic subcellular antigen into an isogeneic host. This maneuver plants reactive sensitized cells in an isogeneic environment to react with allogeneic antigen, and, as such, it is the counterpart of the local passive transfer reactions with sensitized cells and mycobacterial antigens (*31*). In the "irradiated hamster" assay system, sensitized cells admixed with allogeneic antigen are injected into the thin dorsal skin of an irradiated hamster which serves as a sensitive but immunologically incompetent xenogeneic milieu for their interaction (*32*).

As a consequence of recognition of antigen, immunocompetent cells undergo a series of characteristic morphologic changes called blast transformation, which appear to be an expression of delayed-type hypersensitivity (*33*). In this fourth assay system, materials with antigenic activity are detected by their ability to specifically stimulate the blastic transformation of allogeneic cells, as judged by the fact that incubation with allogeneic compared to isogeneic antigen elicits a greater incorporation (by severalfold) of tritiated thymidine into the DNA of the cell (*25*). One study (*34*) represents an attempt to assess antigenic differences among a number of prospective graft recipients by an estimation of the degree to which exposure to subcellular antigen stimulates the transformation of lymphocytes. The results are at best suggestive in that (i) there were only small degrees of stimulation, (ii) the patterns of stimulation were variable, and (iii) the data were not based on the objective label incorporation but rather on the subjective microscopic assessment to quantitate the degree of transformation. The authors concluded that the transformation assay was superior to the alloantibody systems because the latter are based on a limited battery of serums directed against only a fraction of the HL-A determinants. Manson and Simmons (*35*) have induced allograft immunity by exposing cells to allogeneic microsomal membranous antigen in vitro; their work confirms the intimate relation between the events measured in the delayed-type hypersensitivity methods and the induction and expression of transplantation resistance.

Repeated immunization with allogeneic cells leads to the production of alloantibodies. Brent *et al.* (*36*) demonstrated the ease with which the hemagglutination-inhibition technique can be used for the rapid assessment of antigenic activity. Materials were defined as antigens if on prior incubation with antibody they specifically blocked its capacity to cause hemagglutination of donor-type erythrocytes. Inasmuch as the hemolytic and hemagglutination techniques (*37*) depend upon the reactions with histocompatibility antigens located on erythrocytes (a problematic issue, as discussed below), serologic methods have been developed with nucleated cells, including leukoagglutination (*38*), mixed agglutination (*39*), and leukocytotoxicity. The agglutination tests are useful in that they do not depend upon the action of complement, which may be adversely affected by antigenic preparations; nevertheless, the cytotoxicity tests tend to be more sensitive. Techniques for the assay of cytotoxic antibody include phase contrast microscopy (*40*), the

platelet complement-fixation test (*41*), dye exclusion (*42*), susceptibility to lysis by trypsin (*43*), and release of isotopic markers C^{14}, Cr^{51}, and P^{32} from labeled cells (*44*). Methods depending on chromium release are gaining wide application because of their convenience and apparent objectivity. However, all of the cytotoxic techniques are sensitive and relatively reproducible. In recent work with soluble sonicated human antigens (*30*), specific inhibition of monospecific cytotoxic serums could be obtained with 6×10^{-14} mole of antigen, attesting to the high sensitivity of the Terasaki-McClelland phase contrast technique (*40*).

Distribution of the Antigens

Insofar as the available data permit generalizations, transplantation antigens seem to be present on all cells (*45*). They can be found early in ontogeny (*46*). The strong histocompatibility antigens of the various organs cross-react; prior immunization with any tissue induces the accelerated rejection of skin (*47*).

Although the antigens are generally distributed, they appear to be present in varying amounts as determined by dynamic (transplantation) or by static (extraction) methods. For example, transplantation solely across the H-2 barrier in mice reveals that brain and skin induce a stronger immune state than does ovary (*47*). Studies in which antigen has been extracted from a variety of tissues suggest an even more strikingly unequal quantitative distribution of the H-2 antigens. In general, lymphoid tissue has the greatest content of extractable antigen; kidney, lung, adrenal, and liver have moderate amounts; and brain, placenta, and muscle are poorer sources (*47*). The best documented instance of unequal content is liver and spleen. Although studies on both humans and animals have demonstrated that these organs elicit prompt rejection, the potency of splenic microsomal lipoproteins (*48*), solubilized antigens (*49, 50*), and crude homogenates (*51*) is far greater than that of the corresponding preparations from liver. On the other hand, that homogenates of both organs have similar capacities to absorb alloantibodies suggests to some workers that they have an equal content of antigenic determinants (*52*). A more pronounced situation exists with erythrocytes, for prior injection of purified erythrocytes fails to induce immunity, although they are capable of absorbing alloantibody. The apparent differences in the potency of the antigens from various tissues have been attributed to (i) the presence of enzymes capable of catabolizing the antigen (*53*), (ii) the binding or blocking of antigenic sites by other substances in the extracts (*54*), (iii) an unequal distribution of adjuvants, ions, or other cofactors involved in antigen action, (iv) the presence of nonspecific inhibitors of the immune response (*55*), or (v) actual differences between tissues in the chemical expression of the antigenic determinants per se, or of other important regions of the carrier molecule.

Tissue culture cells and media offer a more accessible source material for the extraction of large quantities of transplantation antigens. Haskova and Hilgert (*56*) found that the administration of the ascitic fluid accumulated during the growth of mouse sarcoma I induced immunity in allogeneic hosts. This finding was extended to other tumors, thus yielding a partially purified water-insoluble substance which inhibited specific alloantibody, but which was not tested for biologic activity (*57*). Similarly, insoluble antigen accumulates in the medium when normal cells are maintained in tissue culture. Administration of a membranous sediment of the cell-free medium from 14-day-old cultures of rabbit spleen cells sensitized hosts to reject donor-type skin grafts in accelerated fashion, and exceedingly large, divided doses of this material induced a slight prolongation of graft survival (*58*). Membranous antigenic materal has been detected in the media of human spleen cell cultures by its ability to inhibit the cytotoxic action of specific alloantiserums (*59*). Whether the cell-free antigenic material in these media arises by cell death or lysis, or by constant release of antigen by viable cells remains uncertain.

It has not been possible to isolate transplantation antigen from serum, despite the findings of Shreffler (*60*) that a murine serum globulin is apparently controlled by a portion of the H-2 locus, and of Berg *et al.* (*61*) that the human beta-lipoprotein L_p groups correlate with allograft survival. Searches for antigenic activity in human milk and urine have been unrewarding up to now.

Intracellular Localization

A number of early studies suggested that membranous subcellular fractions (*62*) containing predominantly nuclei (*63*), mitochondria (*64*), lysosomes (*65*), or microsomes (*48*) possess the transplantation antigens. However, present evidence based upon observations with (i) fluorescent alloantibody (*66*), (ii) agglutinating alloantibody (*38*), (iii) antibody absorptions before and after cell rupture (*67*), and (iv) purified fractions (*68, 69*), all indicate that the majority of the strong antigenic determinants are intimately associated with the cell surface membrane. In a recent study employing zonal centrifugation, Popp *et al.* (*69*) were able to isolate a fraction of antigenic particles, thought to be vesiculated fragments of cell surface membrane, just behind the soluble material, but before the microsomes, mitochondria, and cell membranes.

The studies of Pizarro *et al.* (*70*) and of Herberman and Stetson (*71*) have suggested that the antigen gene product of the major histocompatibility locus is a single cistronic unit in the cell membrane. Two recent studies of a number of H-2 systems have demonstrated that the ratios of the activities of several antigenic specificities in subcellular fractions are quite similar (*72, 73*).

Solubilization of the Transplantation Antigens

Solubilization of the antigens from their site on the surface of the cell membrane has become of increased interest owing to the discovery by Medawar (*74*) that nonparticulate transplantation antigens administered intravenously tend to induce prolonged graft survival, and because of the recognition that the antigens are readily amenable to chemical fractionation and analysis only in the soluble state (*75*). The concept of solubilization is based upon the assumptions (i) that it is possible to isolate the antigenic determinants independent of the membranous structure, (ii) that the solubilized product is representative of the determinants on the cell surface membrane, and (iii) that there are no immunologically significant intermediate linkages between the soluble antigenic determinants and the other constituents of the membrane.

Admittedly, it is difficult to compare antigens in the solubilized form with those on the surface of the cell membrane. A great change in potency accompanies cell destruction; all subcellular fractions are far less potent as transplantation antigens than their parent whole cells. The supremacy of viable whole cells over subcellular equivalents has been attributed (i) to their "homing" tendency (*76*), (ii) to their ability to divide, thereby increasing in number and actually providing a higher dosage, (iii) to their content of extremely labile antigenic specificities which are inactivated upon destruction of the cell (*63*), (iv) to their resistance to catabolism upon inoculation into the host (*77*), and (v) to the possibility that cell-bound antibodies are produced much more efficiently against cell-bound forms of transplantation antigens than against the soluble or even the particulate form of these antigens.

There are varying definitions of a solubilized transplantation antigen. Many authors consider their antigen solubilized if it does not sediment on centrifugation at 100,000*g*, that is, if it is free of membrane fragments (*78*). However, in view of the work of Rapaport *et al.* (*79*), this concept is invalid. They found that antigen which did not sediment during centrifugation at 100,000*g* consisted of membrane fragments, as determined by ultrastructural studies on sediments from these "soluble" fractions after centrifugation at 200,000*g*. Others (*80*) have distinguished solubilized from "stabilized" materials on the basis of the supposed chemical complexity of the latter, the alleged requirements of solubilized materials for additional agents for biologic activity, and the sedimentation of "stabilized" preparations in the absence of the solubilizing agent. Most properly this distinction depends upon the relation of the antigen to the solvent. A soluble antigen should be defined as one that exists in true solution in aqueous solvents.

The transplantation antigens have been solubilized with detergents, organic solvents, proteolytic digestion, and sonic energy. Detergents such as Triton, deoxycholate, and sodium decyl and dodecyl sulfate have been employed by a number of investigators. Kandutsch (*81*) has found that extraction of particulate fractions of cells of the mouse ascites tumor sarcoma I with deoxycholate or with 5 percent Triton X-100 yielded an antigenic material, whereas treatment with sodium dodecyl sulfate yielded inactive preparations. After extracting the particulate fraction with the nonionic detergent Triton X-100, Kandutsch and Stimpfling (*82*) solubilized an antigenic material by exposure to snake venom, whose active component was thought to be phospholipase A. Moving-boundary electrophoresis of the water-soluble fraction revealed a major component, the antigen, and a minor component, probably a nucleotide contaminant. Ultracentrifugal analysis of the major component showed that it was highly polydisperse, which suggested to them that the antigen was a collection of polymeric units. However, it is uncertain whether an actual electrophoretic heterogeneity was masked by alteration of the overall charge of the components, because of the presence of lysophosphatide groups after enzymatic treatment. Thus the polydispersity may have represented chemical heterogeneity rather than polymeric forms of a single substance. It is noteworthy that 15 percent of the water-soluble antigen was included in Sephadex G-200, thus indicating an active moiety with molecular size less than 200,000, and that this lower molecular weight component contained only 0.09 percent phosphorus, thereby suggesting a very low lipid content (*83*). The Triton-solubilized materials were true transplantation antigens, capable of (i) inducing accelerated graft destruction, (ii) evoking immunologic enhancement, and (iii) eliciting the formation and inhibition of alloantibody. Immunogenic derivatives of the Triton-solubilized material were prepared by succinylation and by treatment with a mixture of sodium deoxycholate, sodium cholate, and sodium dodecyl sulfate which removed the phospholipid component (*84*).

Although several investigators have been unable to release the transplantation antigens with deoxycholate, Metzgar *et al.* (*85*) have achieved solubilization of the 4^a and 4^b antigenic determinants of the human HL-A system. Splenic and tissue culture cells were disrupted with deoxycholate, which was then removed with magnesium chloride, and a substance was obtained which specifically inhibited agglutination of leukocytes, mixed agglutination, and cytotoxicity reactions and which induced the accelerated rejection of donor skin grafts. Bruning *et al.* (*86*) extracted the HL-B antigens, the products of a less important genetic locus than the major HL-A locus, from the cell membrane sediments of placental tissue with deoxycholate. The antigenic activity appeared to be associated with the protein portion of the lipoprotein material. Manson and Palm (*87*) treated mouse microsomal lipoproteins with sodium decyl sulfate and dodecyl sulfate to obtain a solubilized material which specifically inhibited alloantibody. This material remained dispersed after removal of the detergents, had sedimentation constants ranging from 1*S* and 5*S*, and was retarded when chromatographed with Sephadex G-75. Recently, Kandutsch *et al.* (*73*) extracted the membranous fraction of mouse sarcoma I with 0.25 percent cholate in the presence of 3*M* potassium chloride and obtained an insoluble antigen. Extraction of this antigen with Triton X-114 yielded a solubilized antigenic protein of molecular weight between 100,000 and 200,000.

In a second extraction method organic solvents are used. Morton (*88*) demonstrated that intracellular enzymes could be released and rendered soluble by extraction with butanol. He found that butanol was a more effective solubilizing agent than either autolysis or digestion with lipases, trypsin, or papain in that the intracellular enzymes which he studied were destroyed by proteolytic digestion. Because of its low solubility, butanol saturates aqueous materials without loss of enzymatic activity. It is believed to compete effectively for the polar side chains of the protein, with the alcohol displacing the lipids and thus causing dissociation of the lipoprotein complexes. Kandutsch (*81*) used butanol to solubilize the antigenic activity of a subcellular fraction extracted from water-lysed sarcoma I ascites cells. Manson and Palm (*87*) liberated 40 to 75 percent of the H-2 and "non-H-2" antigenic activity of microsomal lipoprotein with butanol. The soluble material had a reduced lipid content, and existed in a highly aggregated form with an approximate molecular weight of 6×10^6. It induced accelerated allograft rejection when administered subcutaneously with adjuvant, elicited the formation of and the anamnestic rise in H-2 antibody, and inhibited donor-specific hemagglutinins and cytotoxins. Harris *et al.* (*89*) used Triton X-100 extraction and butanol treatment to prepare soluble antigen

from cell membrane fragments of rabbit lymph node and spleen. Subsequent analysis by DEAE-cellulose chromatography and by sucrose gradients indicated that the antigenic material was of a highly complex chemical nature.

Autolytic and proteolytic digestion of membrane fragments of mouse and human lymphoid and tumor cells has been used to prepare histocompatibility substances (*80, 90*). In work on mouse antigens, Davies passed the autolyzate of splenic membrane fragments (prepared by hypotonic elution) over Bio-Gel P-300 and tested fractions from a relatively broad protein distribution pattern for inhibition of immune cytolysis. The active fraction which was partially included in the gel, but which comprised a relatively large portion of the protein effluent, was partially purified by ion-exchange chromatography on DEAE-Sephadex. The antigenic material apparently was of two sizes, the molecular weights being 15,000 and 50,000.

Electrophoresis of the active DEAE component on a continuous polyacrylamide block yielded a single, though relatively broad, zone which, on one of three occasions, showed serologic activity. Although this data was viewed as suggesting homogeneity, more definitive studies are required including data on different *p*H values and pore sizes in the presence of urea or of detergent and results of chemical analysis of the component isolated from polyacrylamide gel. Indeed, using electrophoresis in discontinuous polyacrylamide gel, Jolles and co-workers (*91*) found that the active DEAE fraction from autolyzed cells contained at least three components. Autolyzed and papain-digested histocompatibility substances of human beings were similarly fractionated on Bio-Gel P-300 or on Sephadex G-150 (*78*) and on DEAE-Sephadex to yield glycoproteins with a molecular weight of approximately 50,000, as estimated by sucrose gradient ultracentrifugation. Sanderson and Batchelor (*92*) have treated human spleen cell membranes with insoluble papain because soluble papain (i) acts as a nonspecific inhibitor of lymphocytotoxic serums, (ii) is not completely inhibited by iodoacetate under the conditions usually employed, and (iii) may interfere with subsequent analysis and purification. Using a 20 percent inhibition of the cytotoxic activity of human antiserums as the criterion of antigenic activity, they purified a fraction on DEAE-Sephadex.

There is a good deal of variability in the quantity of antigenic material which can be solubilized by autolysis or papain digestion. Davies states that up to 20 percent of the total number of cytotoxicity units of the membrane fragments can be released by autolysis, and that up to 70 percent may be released by proteolysis with ficin, papain, or bromolein. Nathenson and Shimada (*78*) found that autolysis alone solubilized less than 0.7 percent of the antigenic activity, but that papain solubilized 15 to 45 percent of the activity of the membrane fragments, depending upon the strain of mice from which the cells came. Other workers have obtained far less solubilized antigen with the proteolytic method. Popp *et al.* (*69*) obtained 16 percent solubilization, and Kandutsch *et al.* (*73*) were unable to obtain more than 2 percent of soluble activity from either sarcoma I or from a pool of mouse lymphoid tissues. If the eluted membrane fragments comprise about 40 percent of the antigenic activity of the intact cells, this represents a maximum yield of 0.3 to 18 percent of the total available antigen. Edidin (*93*) feels that proteolysis alone is insufficient to solubilize antigenic materials. By combining chelation and proteolysis with trypsin, he extracted from organs of mouse embryos a glycopeptide which specifically inhibited cytotoxic alloantiserums.

Complex enzyme mixtures such as ficin, papain, and bromolein are highly nonselective in their attack on peptide bonds. Their rate, as well as the location of their cleavage, depend largely upon the primary sequence of the substrate molecule, which may account for the "easy" and "difficult" spleens which were observed by Davies (*90*). Certainly, enzymatic attack on a highly complex and structurally variable cell surface is a "hit-or-miss" approach, especially since the published reports do not mention any attempt to quantitate the reaction, for example, by acid equivalents released during digestion, as would be required to approach reproducibility of the products. Furthermore, the antigenic preparations are not pure enough to warrant any conclusions about the chemical nature of the active material. Indeed, the statement by Davies that treatment of the membrane fragments with agents as different as endogenous cathepsins, ficin, papain, and bromolein yields indistinguishable products is quite surprising.

Products of Sonication

Exposure to sound liberates water-soluble transplantation antigens from mouse spleen, lung, kidney, and liver cells (*49, 75*) and their cell membranes (*94*); from guinea pig spleen, lung, kidney, liver (*25, 50*), and sarcoma (*95*) cells; from dog spleen cells (*22*), and from human spleen cells (*30*) and their tissue culture media (*59*). The effects of sonic (less than 16,000 cycle/sec) and of ultrasonic (more than 16,000 cycle/sec) energy have been reviewed (*96*). Exposure to sound breaks up animal and bacterial cells, as well as molecules in solution, by the generation of heat, by oxidative effects, by mechanical effects including an agitation effect analogous to foaming, and by a frictional effect; however, the most important effect is gaseous cavitation with rapid expansion and violent collapse of the dissolved air within the fluid. The extent of these effects depends upon the intensity and frequency of the sound and upon the physical state of the exposed material.

The activity of the liberated antigens depends upon the conditions of sonication. While potent antigens are released from cells and their membranes by exposure to low-frequency, low-intensity sound, only small quantities of less active material are liberated by high-frequency generators. Thus, Billingham *et al.* (*63*) detected only small amounts of immunogenic mouse transplantation antigen in the soluble fraction, and, similarly, Haughton (*97*) found that antigen was released and then rapidly inactivated after exposure to ultrasound generated at 20,000 cycle/sec with a 60-watt probe. On the other hand, in accord with a large body of evidence (*98*), sonic energy mediated by a diaphragm at 9 to 10 kcy/sec liberates active, water-soluble labile components from intracellular, intraorganelle, or membranous locations. The disparity between the ability of these two forms of energy to liberate active transplantation antigen is probably related to the more pronounced oxidative, bond-breaking, and depolymerizing effects of the probe-generated 60 kcy/sec ultrasound with its propensity toward the development of local heating and the generation of eddy currents. Thus there is a relatively narrow region of intensity in which sonic energy effects solubilization without inactivation of the relatively labile transplantation antigens.

Exposure to the diaphragm-mediated

sound at 9 to 10 kcy/sec liberates 12 to 15 percent of the total immunogenic activity of the disrupted cell. After the debris and cellular membranes are removed by centrifugation at 130,000*g*, the antigenic principle from guinea pigs may be purified by gel filtration on Sephadex G-200 and subsequent discontinuous electrophoresis on polyacrylamide gel to obtain a relatively homogeneous material which is a true transplantation antigen. Such materials (i) specifically accelerate the destruction of donor guinea pig and mouse allografts, (ii) cause more than 60 percent inhibition of human alloantiserums, (iii) evoke the formation of mouse alloantibody (*94*), (iv) elicit direct cutaneous hypersensitivity reactions in already presensitized guinea pig and human hosts, (v) participate in transfer reactions in third-party guinea pig hosts or in irradiated hamsters, and (vi) prolong the survival of renal allografts in dogs (*22*).

The antigen derived from guinea pig tissues is a protein with an R_F of 0.73 to 0.74 (on 7.5 percent polyacrylamide gel, *p*H 9.4) (*50*), and with a molecular weight between 13,000 and 15,000, as estimated by ultracentrifugation with interference optics, by gel filtration in the presence and absence of 5*M* guanidine hydrochloride, and by calculation from the amino acid composition (*99*). This protein is homogeneous when subjected to electrophoresis in gels of varying porosity and in the presence of 8*M* urea. There is no detectable lipid or carbohydrate, with 1 percent level of sensitivity of the method. The amino acid composition is characteristic and reproducible: there are many serine and threonine residues and numerous acidic amino acid residues, which is consistent with a high electrophoretic mobility at *p*H 9.4. There are few aromatic amino acids, and sulfur-containing amino acids are probably either low in number or absent. All these general features resemble the amino acid composition of blood group substances (*100*). Of interest are the distinct and reproducible differences in the content of serine, alanine, isoleucine, leucine, and valine—and, possibly, in tyrosine and phenylalanine—between the antigens prepared from histoincompatible lines of guinea pigs. By the use of similar techniques, an electrophoretic component with HL-A antigenic specificities was found to have an R_F of 0.78 to 0.80 (*30*), a molecular weight of 34,600, lack detectable carbohydrate or lipid, and similar general features of its amino acid composition. Such comparable data lend support to the concept that the strong transplantation antigens of numerous species are homologs.

Although the antigenic specificities are associated in the same fraction of the cell membranes, it is uncertain whether they all reside on the same molecule. Solubilization results in the liberation of most of the known antigenic determinants (*30*). However, it is uncertain whether the determinants when liberated are organized as they exist on the cell membrane. From analyses of the chemically complex products obtained by proteolytic digestion of membrane fragments, some workers have claimed that the antigenic specificities of the strong histocompatibility loci can be separated by ion-exchange chromatography (*90*). However, these individual specificities have neither been isolated nor chemically characterized. Indeed, it is possible that these apparently separable antigenic specificities represent but fragments whose chemical structures are overlapping or partially identical, resulting from variable sites of enzymatic cleavage. The chromatographic separation of these fragments would then be due to variable aggregation or charge of the antigenic units. On the other hand, there is evidence that some of the specificities are present on a single molecule. Davies (*90*) reacted the crude autolyzed antigen from an F_1 mouse donor with an alloantiserum specifically directed against one of the parental strains, and then isolated the antigen-antibody complex by gel filtration. He found that this complex contained the antigenic specificities of the other parent and concluded that both phenotypes were present on a single molecular species. Thus the molecular distribution of the antigenic specificities remains uncertain, only to be ascertained by further studies with a variety of purified, monospecific serologic reagents and of homogeneous antigenic molecules.

Chemical Nature of the Antigenic Determinant

The pioneering experiments of Billingham *et al.* (*63*) demonstrated that a nuclear subcellular fraction was able to immunize recipients against subsequent grafts. Since deoxyribonuclease but not ribonuclease or trypsin inactivated the preparation, they suggested that DNA determined transplantation specificity. However, Haskova and Hrubeskova, as well as Medawar, were subsequently unable to elicit accelerated rejection with purified DNA (*101*). Furthermore, Castermans and Oth (*102*) found that extraction of the homogenate of the nucleus with sodium chloride yielded an active supernatant that did not contain DNA, and an inactive sediment that did contain DNA, thus proving that a component other than DNA carries the antigenic specificity.

Later work by Billingham *et al.* (*103*) demonstrated that the antigenic activity was present in the sediment of cells that had been exposed to ultrasound and centrifuged at 27,000*g*. They proposed that the determinant was a mucoid because there was drastic impairment of the biologic activity after exposure to two reagents: (i) receptor-destroying enzyme, a complex mixture from *Trichomonas foetus*, and (ii) periodate (0.005 to 0.01*M*). Several other investigators have demonstrated that transplantation antigens are inactivated by periodate (*104, 105*). For example, Sanderson (*106*) found a 70- to 80-percent reduction in the inhibitory activity of his papain-solubilized human antigens for the LA 2 but not for the other LA specificities after they had been exposed to 0.01*M* periodate; he interpreted this result to mean that LA 2 may be mediated by a carbohydrate-dependent configuration and may be distinct from the other antigenic determinants. However, none of these studies have demonstrated specific effects, such as oxidation products, on the carbohydrate moiety. On the other hand, Kandusch (*83*) has shown that, after mouse transplantation antigens are exposed to 0.001*M* periodate, there are changes in the content of several amino acids including tyrosine, isoleucine, and leucine; these same amino acids have been implicated in guinea pig transplantation allotypy (*99*). However, it is difficult to ascribe the nature of the antigenic determinants either to protein or to carbohydrate on the basis of the treatment of highly complex mixtures with relatively nonspecific oxidizing agents such as periodate.

Brent *et al.* (*36*) found that some polysaccharides with Forssman affinities —blood group substance A (but not B, H, or Le^a)—type XIV pneumococcal polysaccharide (but not types I, II, or V), and *Shigella shigae* polysac-

charide, inhibited the agglutination of erythrocytes by alloantiserums in a fashion analogous to the hapten inhibition observed with blood group isoantiserums. In later studies Davies (*105*) found that D-galactopyranose-β-(1 $\rightarrow$ 4)-D-glucosaminoyl residues partially inhibited alloantiserums against specificity H-2 18 (R) and that *N*-glycolylneuraminic acid specifically inhibited some mouse alloantiserums. However, this line of investigation has failed to yield insight into the chemical nature of the determinants, since, in general, these are very weak effects.

Intensive efforts by several investigators (*48, 107*) suggested that water-insoluble materials containing approximately equal proportions of lipid and protein and with a low carbohydrate content mediate transplantation immunity. These lipoproteins (i) induced accelerated graft rejection, (ii) elicited the formation of specific alloantiserums, and (iii) inhibited the reactions of these serums in vitro. Davies (*105*) proposed that the lipid moiety either carries or determines the transplantation antigenic specificity since he found that the protein precipitate after extraction with organic solvents was inactive, and since there was an increasing proportion of lipid with increasing degrees of purification. However, it is now clear that more purified materials containing little or no detectable lipid possess all of the attributes of transplantation antigens (including immunogenicity), and that lipoidal fractions obtained by chloroform-methanol or ethanol extraction have no antigenic activity (*25, 50, 75, 84, 99*).

Indeed most of the evidence suggests that polypeptide is essential for antigenic activity. First, Kandutsch and Reinert-Wenck (*104*) noted irreversible destruction of antigenic activity after exposure to conditions that cause protein denaturation: 50 percent urea, 90 percent phenol, aqueous alcohol, heat, and *p*H values less than 4 and greater than 9. Second, Kandutsch (*83*) has shown that the proteolytic enzyme pronase degrades antigenic material to an inactive, lower molecular weight substance, a suggestion that the previously observed resistance of crude fractions to other proteolytic enzymes may have been due to an inaccessibility of the agent to the active region of those antigens. Furthermore, deoxycholate treatment and succinylation, which affect protein configuration, partially destroy the immunogenic properties of extracts solubilized by detergents (*84*). Third, the mediation of transplant rejection by a cell-bound immune response implicates polypeptide specificities since, as Holborow and Loewi (*108*) have summarized, "there is practically no evidence that man or animals develop delayed hypersensitivity toward polysaccharides, and in that respect they differ sharply from proteins." Fourth, chemical analyses of homogeneous transplantation antigens (*99*) strongly suggest that their determinants depend upon protein structure, because (i) they show allotypic differences in their primary protein structure (amino acid compositions), and (ii) they do not contain either carbohydrate or lipid at the 1 percent limit of the analytical method. Thus in the case of guinea pig antigen (molecular weight 15,000) there is at most one residue, and in the case of human antigen (molecular weight 34,600) there are at most two residues of carbohydrate per molecule. Although it is unlikely that carbohydrate, if indeed present, represents the major determinant of these antigens, one cannot absolutely rule out the possibility that a single carbohydrate residue could confer a unique protein configuration that determines antigenic specificity.

The polymorphism of the primary protein structure of these antigens is strikingly similar to that observed in rabbit immunoglobulin light chains (*109*). In both cases, there is a correlation between allotypic specificity and the amino acid composition of the polymorphic substances. In the rabbit, at least four genes at the *b* locus control structural features of the light chains. Amino acid differences between individuals having different allotypic specificities suggest that the *b* locus controls at least part of the amino acid sequence of these proteins. The transplantation antigens may form an analogous system, for these antigens are products of alleles distributed among some but not all members of the same species and may thus be considered to form an allotypic system (*110*). Although not all of the observed amino acid differences may be related to antigenic specificity, it is probable that some of them either determine the primary antigenic configuration per se or determine pertinent differences in the specific secondary and tertiary structure of these transplantation antigens.

Summary

As readily distinguishable products of well-defined genetic systems, transplantation antigens are marker substances for studies on the relation of genes to their products and on cell membrane physiology. According to most of present evidence, the antigens represent the direct polypeptide transcriptions of the histocompatibility loci. Further studies will be required to determine the role of these substances in cell membranes and thereby to elucidate the nature of the recognition phenomenon which is experimentally challenged by allotransplantation.

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