Immunological Tolerance in Organ Transplantation

Fair Prospect or Fanciful Folly?

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SUMMARY
A formidable array of practical problems must be solved at laboratory level before tolerance induction in organ transplantation can be considered a realistic clinical possibility. I believe it is too early for even tentative human experimentation in this field. However, the work of the last decade has shown unequivocally that even adult animals can readily be rendered tolerant of even exceedingly powerful antigens. The principle of tolerance has such great specificity and such compelling elegance when compared with present-day aids to organ transplantation that intensive effort must go into harnessing it to clinical use. It will certainly enter the clinical homograft scene within a decade, and xenografts are inconceivable in its absence.

TWENTY YEARS AGO, Burnet and Fenner recognized that a discrimination between "self" and "not self" was a fundamental duty of the lymphoid system and predicted that the system could be "tricked" into tolerating foreign antigens under some circumstances. Five years later, Billingham and associates made immunological tolerance into an experimental fact and ushered in the modern era of transplantation biology. Since then, a mountainous literature on immunological tolerance has accumulated; yet no evidence exists to suggest that any living transplant recipient has been rendered truly tolerant of his homograft. He does tolerate his graft, but only with the aid of continuous administration of immunosuppressive drugs.

Despite the exciting recent progress in clinical use of organ grafts, the present state of transplantation immunotherapy can hardly be described as satisfactory. From the practical point of view, each of the three main agents—corticosteroid drugs, azathioprine, and antilymphocyte globulin or ALG—has its own special toxicity, and the use of them in combination seriously diminishes the recipient's capacity to cope with his microbial environment. From the theoretical point of view, the blunderbuss approach lacks elegance, in that none of the treatments can be considered specific for the actual immune response that needs to be checked.

What is immunological tolerance? It can be defined as a specific reduction of an animal's capacity to respond to an antigen, brought about by prior exposure to the antigen. The goal of tolerance in organ transplantation is indeed an admirable one—to remove the host's capacity to respond to antigens of the graft but to leave intact all other immune potential. It can be thought of as the deletion of...
one specific word from the immunological dictionary of the individual. While ALG is the most specific immunosuppressive (or should be!) in the sense of attacking lymphocytes only and not bone marrow stem cells, or other dividing elements, tolerance has a specificity of an altogether different order. Its induction should not harm the host in any way and quite definitely should not diminish immunity against infections.

**History of Tolerance Induction**

The early history of tolerance induction dealt mainly with the injection of living cells into embryos or very young animals. It was found that lymphoid cells were particularly efficient in inducing a later tolerance to skin homografts of the donor strain. This proved to be due to two causes: First, donor lymphoid cells colonized the host and lived for long periods, constituting a depot source of antigen. Secondly, lymphoid and reticuloendothelial cells are particularly rich in transplant antigens. However, the use of lymphoid cells carried with it a great hazard: as the donor cells were themselves immunologically competent, they could mount an immune attack on the host—the so-called graft-versus-host (GVH) response. Unfortunately, this frequently killed the recipient. Clearly, therefore, for a number of reasons this form of tolerance induction is of no interest to us in a human transplantation setting.

The next phase in tolerance induction was concerned chiefly with an extension of the above type of procedure to adult animals that had been rendered temporarily incapable of responding to antigens through the use of whole body x-irradiation or cytotoxic drugs. In general, a satisfactory degree of tolerance could again be induced only through the use of living cells. Thus, we encountered the same problems of GVH disease, only accentuated on this occasion by the inherent toxicity of the drugs or treatments used. There was a brief vogue for the use of fetal cells to avoid GVH reactions, but no really satisfactory protocol of relevance to the human situation emerged.

The third phase of tolerance research is the one in which we are now engaged. It became clear that this powerful principle could be harnessed to good use only once it had been more fully understood. Thus, attention was turned to the induction of tolerance not toward organ grafts but toward pure, defined antigens which could act as model substances and could allow detailed analysis of the influence of parameters such as antigen size, dose, persistence, distribution, organ concentration, and chemical constitution. The long-range hope of this field is that as knowledge of the chemical nature of transplant antigens advances, information gathered through the use of these model antigens can rapidly be applied clinically. It is recognized that the destruction of homografts depends on a combined effect of a cellular immune attack of delayed hypersensitivity type and anti-graft antibodies. Current tolerance models are directed chiefly at the latter, but it is likely that the key principles which are at work will apply to both types of response.

**The Various Categories of Lymphoid Cells**

The best understanding of tolerance can be reached when one thinks of the immune response of an animal as the integration of a large number of events occurring between individual antigen molecules and individual lymphoid cells. It is therefore important for us to consider the nature of the antigen-reactive lymphocytes, as well as the nature of antigen-trapping mechanisms in the body.

The lymphocytes which respond to antigen are to be found in the blood, the lymph, and the peripheral lymphoid organs (lymph nodes, spleen, Peyer’s patches, tonsils, and appendix). The peripheral compartment of the lymphoid system must be thought of as a highly dynamic one, with cells constantly on the move between the different individual organs. Lymphocytes born in lymph nodes or spleen enter the circulation via the lymphatics, but also display a marked “homing” tendency—a desire to return to lymphatic tissue, so a particular lymphocyte may complete this cycle many times.
Where do these lymphocytes arise originally? It used to be thought that they differentiated from reticular cells in the lymph nodes themselves, but this idea has been proven quite wrong. All lymphoid cells in the peripheral compartment of mammals stem from two sources, the bone marrow and the thymus. Bone-marrow derived cells are chiefly responsible for antibody production. Thymus-derived cells are mainly involved in cellular immune reactions of delayed hypersensitivity type. However, the distinction is by no means an absolute one, and in some immune responses the two cell lineages act collaboratively. The generation of lymphoid cells in bone marrow and thymus is independent of antigenic stimulation. It is regulated by a variety of inducers, including particularly a humoral factor manufactured by thymic epithelial cells. In other words, these organs are antigen-independent factories for various kinds of lymphocytes. They will pour out their product just as effectively in germ-free as in conventional animals. They are not factories for antibodies as such. The peripheral lymphoid system stands in complete contrast. In fact, cells in lymph nodes, spleen, and so forth divide only after antigenic stimulation. It is to the peripheral compartment that we must look for the real, antigen-mediated action. Here the executive immune responses occur on antigenic command; here we must endeavor specifically to turn them off by induction of tolerance.

The population of lymphoid cells in the peripheral compartment is heterogeneous from a number of viewpoints. First, we must differentiate between virgin cells and committed cells. Virgin cells are those that have entered from one of the two basic lymphocyte factories (bone marrow or thymus) but have not yet been stimulated by antigen. Committed cells comprise the majority of cells in the peripheral compartment of normal adult animals. They are cells that have gone through one or more cycles of antigen-induced mitosis. The committed cells in turn can be broken into three main types: antibody-forming cells, effector cells of cellular immunity, or memory cells. Antibody-forming cells are plasma cells and modified lymphocytes which secrete humoral antibody into the circulation. Effector cells of cellular immunity are lymphocytes which seek out antigen depot sites, for example, organ transplants, and there cause inflammation and tissue damage. Memory cells are lymphocytes which do not themselves form antibody but which represent the progeny of certain virgin antigen-reactive cells and have the property of reacting vigorously to a secondary antigenic challenge.

To sum up, an antigen on entering the body can encounter and react with virgin antigen-reactive lymphocytes (which may be bone marrow or thymus-derived) and memory antigen-reactive lymphocytes. The result of such an encounter will normally be blast cell transformation of the antigen-reactive cells. This is followed by mitotic divisions leading to the production of the three categories of committed cell. It is this clonal development which we must seek to forestall by tolerance-induction treatments.

One of the great problems in immunology is that most of these different types of cells look very similar, even under the electron microscope. Thus the heterogeneity is a subtle, functional one which does not confer morphological differences. A final and extremely important point in considering the nature of lymphoid cells is a heterogeneity in their reactivity to different antigens. If one takes a population of normal thoracic duct lymphocytes and assesses their ability to respond, for example, to Salmonella flagellin, we find that only one cell in 50,000 in the population can react and produce a clone of antibody-forming progeny. Even considering that many of the lymphocytes are committed cells, this is a small proportion. Further, if two unrelated antigens are used, they appear to address different populations of antigen-reactive cells: antigen A speaks to lymphocyte population A; B, to B and so forth. We do not know just how restricted in its response potential each virgin cell is; some authors favor the view that such cells can express only one
potential; others, the notion that the cell is toti-potent but only expresses one or a limited range of reactivities at a given time. Most modern authors appear to concur in the view that the central event in immune induction is a union between an antigenic determinant and a receptor site on the surface of the antigen-reactive cell. The receptor is believed to be antibody, and thus there now appears to be agreement on the concept that the genetic potential for antibody-production does exist within unstimulated animals but only needs antigen to trigger its expression.

In Vivo Effects of Antigen

We must now consider exactly how antigen entering the body can affect the corresponding population of antigen-reactive cells. So far, we have commented only on possible stimulation. It is essential to note that antigen can also do the reverse. It can cause an inactivation of the lymphocyte so that the total pool of antigen-reactive cells with respect to that antigen is depleted. Under what circumstances is antigen likely to do this? Four interrelated factors are chiefly concerned: dose, persistence, molecular weight, and immunogenicity. We must consider each in turn.

Two Zones of Antigen Dosage Capable of Inducing Tolerance

It has been known for over 20 years that an excessive dose of antigen can paralyze the immune system. It has been clearly realized only since 1964, however, that the repeated injection of small and in fact sub-immunogenic amounts of antigen into normal adult animals can lead to tolerance. There are thus two zones of antigen dosage which can cause tolerance separated by a broad dosage zone which causes immunization. The actual amount of antigen needed to achieve, respectively, low-zone tolerance, immunity, and high-zone tolerance will vary with different antigens. For example, bovine serum albumin (BSA) injected repeatedly into mice causes low-zone tolerance with 10 μg, and high-zone tolerance with 10 mg, the immunity hump being in between. With flagellar antigens in rats, low-zone tolerance can follow daily doses of 10⁻⁷ μg, or 100,000,000 times less than in the BSA system; high-zone tolerance requires from 10⁻⁴ to 1 μg depending on the detailed protocol. In other words, with some antigens high-zone tolerance can be caused by less material than is required for low-zone induction with others. The two zones of tolerance can be demonstrated in both newborn and adult animals, though tolerance is usually more complete with injection schedules that begin at birth.

How can such an apparently strange dose-response profile be understood? It is vital to appreciate that the development of an antibody-forming clone depends on a continuing effect of antigen. There are believed to be six to eight sequential mitotic divisions involved, and in all probability all except the last one to two cycles require the presence of antigen at the lymphoid cell surface. Let us take a situation in which a very low concentration of an antigen is present. A cell may be "hit" by antigen and may embark on the first steps toward antibody production. However, it may, in view of the low concentration, not encounter antigen again. This abortive activation could lead to death of the cell. A wide range of intermediate concentrations of antigen could cause a hit frequency of the right nature, leading to immunization. Finally, a very high hit frequency could once again lead to failure of correct clonal development. Supra-optimal saturation of surface-binding sites could prevent even the initial transformation.

Persistence of Antigen

Obviously this concept is closely allied to dose. Some antigens are rapidly cleared from the extracellular fluids through a combination of phagocytosis, catabolism, and excretion. Clearly such materials would have to be administered repeatedly to achieve a high likelihood of reaching most or all antigen-reactive cells. Other antigens, such as heterologous serum proteins, might persist for long periods following a single injection. A detailed kinetic study of the effect of persistence.
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of antigen on tolerance induction\(^{13}\) has shown that high-zone tolerance can be induced within a few hours, but induction of low-zone tolerance takes some weeks. This fits in well with our concept of hit frequency as an important parameter. At low concentrations of antigen, the statistical chance of a particular cell's receiving even a single hit in a given time is low. Therefore, a considerable time must elapse before a sufficient proportion of the cells reactive to that antigen have been effectively modified.

**Molecular Weight of Antigen**

In adult animals, the molecular weight of an antigen has a profound effect on its in vivo action. This is perhaps best exemplified by an example taken from work with the model antigen, Salmonella flagellin.\(^{16,17}\) This is the so-called H antigen and is available in a number of pure forms. Polymerized flagellin (POL) consists of linear aggregates of flagellum-shaped, rodlike particles. The actual protein constituent in monomeric form is flagellin, mol wt 40,000 (MON), and this can be broken down further into smaller fragments, of which one, fragment A (mol wt circa 20,000) retains all the antigenic determinants. We thus have a hierarchical series of model antigens displaying the same specificity available in forms of progressively lower molecular weight. As one goes down the molecular weight scale, tolerogenicity (tolerance productivity) increases; as one goes up, immunogenicity increases. Thus, POL is powerfully immunogenic and can only cause tolerance under special circumstances. Fragment A is powerfully tolerogenic and can only immunize if adjuvants or multiple injection schedules are used. In other words, with a single injection of an equal amount injected once, POL and fragment A achieve diametrically opposite results in vivo, and MON falls in between.

How is this influence of molecular weight mediated? At this stage we do not know what proportion of the effect is due to differences in antigen handling by the reticuloendothelial system (RES) after antigen injection, and what proportion is due to a direct effect of the size of the antigen molecule or fragment which actually impinges on the surface of the reactive lymphocyte, but we suspect that the former factor is very important. Antigens that are avidly taken up by macrophages tend to be better immunogens. Those that remain largely extracellular, either in the extracellular fluid compartment or attached to the surface of cell processes in lymphoid follicles\(^{16-18}\) tend to be better tolerogens. Whether these observations are causative or coincidental remains to be determined.

**Immunogenicity**

Even when all of the three factors considered, dose, persistence, and molecular weight, have been discounted, there is still a huge variation in the relative in vivo effects of various foreign materials. For example, the incredibly small dose of \(10^{-8}\) \(\mu\)g (\(10^{-15}\) g) of the somatic polysaccharide antigen of Salmonella can cause antibody formation, this dose representing approximately 1,000 molecules injected.\(^{19}\) By contrast, an immunogenic dose of a heterologous serum protein, such as bovine serum albumin, would be 1 mg, or a trillionfold more. The broad rule appears to hold that those types of antigens which are good immunogens can, if prepared in soluble, low molecular weight form, be excellent tolerogens as well. In fact, surprisingly good tolerance can be achieved by injections of sub-picogram amounts of flagellin. Thus, old notions of an overwhelming paralysis through antigen overloading must be discarded. Why there should be such huge inherent differences in the in vivo immune properties of various foreign molecules is still obscure. However, the principles outlined should give us cause for hope rather than despair. From all we know, histocompatible antigens are powerful ones, and thus powerfully tolerogenic preparations should become available in due course.

**Tolerance Induction in Vitro**

Recent work in our laboratory,\(^{20,21}\) indicates that the initial events of tolerance induction
can occur in vitro, and even in the cold. Normal mouse spleen cells mixed in vitro and left at 4°C for 6 hr with POL in doses adequate for high-zone tolerance induction fail to form antibody when subsequently exposed to immunogenic doses. While this is looking far ahead, such a process might one day be possible in transplantation. One could envisage circulating lymphocytes being exposed to antigen in vitro and being rendered tolerant before the blood is returned to the body. Of far more immediate significance is the fact that an in vitro model of tolerance induction should allow us to obtain a much more detailed picture of the actual cellular and biochemical steps involved in tolerance.

Effect of Immunosuppressive Drugs
The chief effect of immunosuppressive or cytotoxic drugs in tolerance induction appears to be to reduce the possibility of antibody production, which indirectly aids tolerance induction but may not materially reduce the required antigen dosage. At the single cell level, tolerance and immunity can be regarded as competing probability functions, and if the possibility of one phenomenon is reduced, the balance is swung in favor of the other. The elimination of the proliferative events of immunity is helpful in another way as well. Memory cells, once born, are more difficult to render tolerant than virgin cells. So, in a sense, "immunity begets immunity" and this is probably why tolerance is not a more frequent total consequence of antigenic stimulation.

Practical Problems in Tolerance Induction in Organ Transplantation
These can be discussed under the following main headings: (1) purification and characterization of transplant antigens; (2) duration of tolerance; (3) source of antigen; and (4) risk.

Purification and Characterization of Transplant Antigens
Considerable progress has been made in the purification and characterization of mammalian, and particularly mouse, transplantation antigens. Some major antigenic factors, such as mouse H-2 antigens are available in particulate, semi-purified form as lipoprotein, cell membrane fractions, and also as soluble molecules. The latter have been described in various molecular forms varying from 7,000 to 50,000 in estimated molecular weight. While these fractions are some thousands of times purer than the original crude cell homogenate from which they were derived, they still cannot be regarded as fully purified. This is currently an area of rapid progress.

Characterization of in vivo effects of transplant antigen preparations is much less far advanced. In what dosage will they cause low-zone tolerance, immunity, and high-zone tolerance? This is an exciting field for further study. We take some hope from the work of Owen and associates. With quite crude antigen preparations injected daily for 5 weeks, significant prolongation of kidney homograft survival was obtained. Moreover, the daily dosage represented material from only 2,500 donor liver cells. In other words, picogram quantities of antigen probably sufficed. This shows how full of potential promise this research area is.

Duration of Tolerance
Experience with model antigens shows that low-zone tolerance is very transient, but that high-zone tolerance can be long lasting, under some circumstances lasting a lifetime. From this viewpoint, high-zone tolerance would be a preferable state in organ transplantation. Until more information is available on dose-response kinetics using purified or semi-purified histocompatibility antigens, the question of whether high-zone tolerance toward organ grafts would ever be feasible cannot be answered. We must also bear in mind that many genes control histocompatibility. While H-2 in the mouse or its equivalent HLA in the human represents the most powerful antigen, all the minor loci contribute to the total immunogenicity of a transplant. Little is known of the chemistry of non-H-2 antigens. Should the dose-response profiles for the different components vary greatly, and antigen preparations be impure, we might be faced with
the troublesome situation where a protocol designed to achieve tolerance toward one component actually causes immunization against another. This dilemma can only be avoided by rigorous antigen purification and better understanding of the minor gene loci and their products.

**Source of Antigen**

If low-zone principles can be applied, the antigen source is less of a problem than if high-zone dosage must be used. Should one desire to induce tolerance before operation, the problem arises that low-zone tolerance takes some weeks to achieve, and thus the prospective donor would have to be available well before the operation. This places us in the realm of living human volunteers or xenografts. Peripheral leukocytes or a liver biopsy would provide sufficient material. With high-zone tolerance, effects can be achieved much more quickly, and the use of cadaver material could be considered. A more practical approach may be to think of the tolerance course as being concurrent with the post-transplant convalescent period and as being accompanied by an immunosuppressive regime. Here the picture is clouded by the probability of the release of unknown quantities of antigen from the graft itself. Again, this would be less of a worry with high-zone tolerance procedures.

One major hope is that we will learn enough about the chemistry of transplant antigens that their synthetic manufacture could be contemplated. At present this looks a remote possibility, but Davies has pointed out that antigenic systems which look impossibly complex when considered in purely serological terms often become much simpler when the chemistry underlying the various antigenic factors is understood. A good example is the Kauffman-White scheme of Salmonella O antigens. It may well be that when our knowledge of transplant antigens is sufficiently advanced for this to be feasible, matching procedures will be so effective as to make induction of tolerance much less important. However, tolerance may well be the principle to cope with minor antigens and here perhaps semi-purified, mixed preparations will suffice.

Once again xenografts must be briefly mentioned. If these ever become practicable, inbred lines of primate donors could be developed and in that case tolerance induction, using purified and even synthetic antigens, could well be a necessary part of treatment.

**Risks**

Every tolerance regimen carries with it the risk of some immunization. All the literature on tolerance shows the reality of this problem. Different animals in a given treatment group vary and so do animals of different genetic strains. This variation is more prominent in the low zone. In a transplant setting, the results of causing antibody formation by a tolerance course would be a disastrous, "white graft" type hyperacute rejection. This is one further reason why tolerance induction should proceed under a cover of immunosuppressive drugs, and perhaps should accompany, rather than precede, the operation.

**References**


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