Lymphocyte Stimulation: Selective Destruction of Cells during Blastogenic Response to Transplantation Antigens

Abstract. *The blastogenic response of human lymphoid cells toward any individual's transplantation antigens can be deleted by the addition of tritiated thymidine of high specific activity during the incubation of the lymphoid cells in mixed leukocyte culture. After the immunocompetent clones which responded to histocompatibility antigens had been destroyed, the remaining population still retained its capacity to respond to unrelated antigens, including other transplantation antigens.*

The primary immune response depends upon the proliferation of immunocompetent cells which occurs after initial exposure to antigen. Indeed, measurement of the synthesis of DNA by incorporation of isotopically labeled thymidine is a useful technique for the evaluation of proliferation of immunocompetent cells in vitro. In the mixed leukocyte culture (MLC) reaction, incorporation of labeled thymidine reflects the proliferation of lymphocytes responding to the disparate antigens present on the cells from unrelated donors (*1*). The significance of this response was clarified when Amos and Bach demonstrated that the stimulation in one-way human MLC could be accounted for on the basis of incompatibilities in the HL-A phenotypes of the donors (*2*).

Thus, cells from identical twins and from siblings with identical HL-A types do not proliferate in MLC, while cells from siblings with differing HL-A antigens will be stimulated. In this context, the MLC reaction has been used as a guide to histocompatibility, especially in clinical attempts to transplant immunocompetent cells in circumstances where the graft-versus-host reaction is a potential problem (*3*). We now have evidence that clones of human lymphoid cells within a population can be selectively deleted in one-way MLC, so that the resulting population displays a type of "tolerance" to the transplantation antigens of the inactivated leukocytes of a histoincompatible donor. Subsequent challenges of the "tolerant" population with inactivated cells bearing unrelated transplantation antigens, or with soluble antigens or mitogens, still result in a wave of new DNA synthesis, whereas reexposure to the initially inactivated cells does not.

The technique developed to accomplish this deletion of response to transplantation antigens is based on the observation of Dutton and Mishell (*4*) that the plaque-forming antibody response of murine spleen cells to sheep erythrocytes could be abolished by exposing the spleen cells to tritiated thymidine (^{3}H-T) of high specific activity 24 hours after they had been exposed to the heterologous erythrocytes. Only the cells that are stimulated by the antigen participate in DNA synthesis and incorporate the compound; because of the high specific activity and the short irradiation path of tritium, only these antigen-stimulated cells destroy themselves by incorporating a lethal dose of the compound in a form of "tritiated thymidine suicide."

Suspensions of human peripheral leukocytes (1×10^6 cell/ml) from each donor were prepared for two-way MLC and for one-way MLC with the mitomycin C technique (*2*). The two populations of cells were washed in a balanced salt solution and incubated in minimal essential medium (Spinners) supplemented with fetal calf serum (10 percent) and antibiotics. After 24 hours of culture at 37°C, 10 μc of ^{3}H-T of high specific activity (18 c/mmole) were added to each 2-ml culture; the cells were incubated for 18 hours in the presence of the labeled nucleoside, washed twice by centrifugation, and suspended in fresh medium in the presence of a test antigen or mitogen. Test antigens included mitomycin-treated cells from the same donor whose inactivated cells were used in the original culture or from a different donor, or keyhole limpet hemocyanin (KLH); the plant mitogen used was phytohemagglutinin (PHA). Cultures were exposed to test antigens for 5 days; those exposed to PHA were harvested after 3 days. Inasmuch as ^{3}H-T had been used for the initial killing procedure, [^{14}C]thymidine (30 mc/mmole) was used to assess lymphocyte stimulation; 0.1 μc was added to each culture 24 hours before the cells were harvested. Samples were prepared for autoradiography or precipitated in cold, 10 percent trichloroacetic acid and prepared for scintillation counting (*5*).

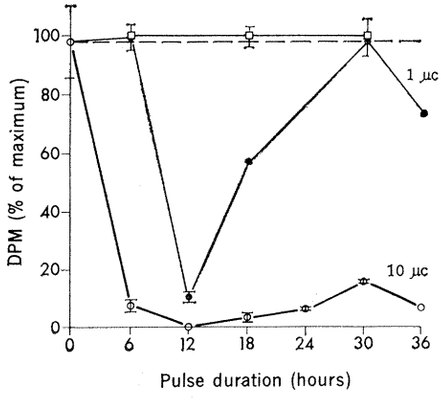

Fig. 1. Effects on [^{14}C]thymidine incorporation in MLC of [^{3}H]thymidine treatment of mixed leukocytes. ^{3}H-T treatment was begun 24 hours after initiation of the cultures. The durations of the ^{3}H-T treatment are plotted on the abscissa; cultures were harvested 5 or 7 days later. The amount of [^{14}C]thymidine incorporated in untreated MLC of the donor pair used in this experiment was defined at 100 percent (- - -) and ^{3}H-T–treated samples were related to this standard. (○) Cells treated at various times with 10 μc of ^{3}H-T; (●) those treated with 1 μc of ^{3}H-T; (□) cells treated at various times with 10 μc of ^{3}H-T in MLC and subsequently exposed to PHA; (I) ± S.E.M.; *DPM*, disintegrations per minute.

The effects of the treatment with ^{3}H-T were first studied in two-way MLC. In these studies, 10 μc of ^{3}H-T was added 24 hours after the cultures were initiated and incubation was continued for 18 hours. The ^{3}H-T was washed out, and incubation was continued. The blastogenic response normally seen 5 to 7 days after exposure to a test antigen was completely abolished, although cell viability was only slightly less than in control cultures. Autoradiographs prepared at the end of the ^{3}H-T treatment period showed labeling limited to the nuclei of lymphoid cells; less than 1 percent of the lymphocytes were labeled. The MLC's that had been incubated for 3 to 4 days after the ^{3}H-T treatment contained some blast forms; however, these transformed cells appeared disrupted and were nonviable when evaluated with the trypan-blue exclusion test. Shorter durations of exposure (6 hours) or lower concentrations of ^{3}H-T (1 μc) for 18 hours did not consistently abolish the entire MLC response (Fig. 1). Consistent results were obtained with 10 μc of ^{3}H-T; those obtained with 1 μc varied both within and in different experiments. The variation cannot be explained simply on the basis of degra-

dation in vitro on the ^{3}H-T or the response of late reacting clones. In view of this variability at the 1 μc dosage, 10 μc was chosen as the standard amount of the isotope for induction of "tritiated thymidine suicide."

When PHA was added after the ^{3}H-T was washed out of the MLC and the leukocytes were incubated for 3 days more, the [^{14}C]thymidine incorporation that occurred was less than that in untreated (no ^{3}H-T) control cultures that had been incubated with the mitogen, suggesting that the exposure to ^{3}H-T had induced some diminution in overall responsiveness.

Studies with donors of different HL-A phenotypes were then done in one-way MLC combined with the ^{3}H-T killing technique. Elimination of the one-way MLC response of active leukocytes from any first donor (A) by ^{3}H-T treatment after A's leukocytes had been exposed to inactivated ($_i$) leukocytes from a second donor (B_i) did not affect the normal MLC reaction when the treated A cells were exposed to inactivated leukocytes from a third donor (C_i) (Fig. 2). In each series of experiments, an individual donor's cells were tested as A, as B_i, and as C_i, thus eliminating the possibility that the response after ^{3}H-T treatment was due to any one cell population being a stronger stimulant.

Positive and negative controls for each procedure were used in both two-way and one-way MLC. All samples in each experiment, including controls, were subjected to identical washing and medium-changing steps, eliminating the possibility that cell interactions, variations in medium, or other events unrelated to the lethal incorporation of ^{3}H-T itself accounted for our observations. Routine controls, wherein additional inactivated cells (B_i or A_i) were added to the culture after the ^{3}H-T treatment, did not induce an MLC response, an indication that the deletion was specific in the population of A cells that had initially been exposed to B_i. The routine controls with B_i also exposed ^{3}H-T–treated A cells to a higher total concentration of B_i as compared to the direct studies with a mixture of A and B_i cells, and showed that a normal response could not be restored by doubling the dose of the stimulatory antigen (B_i) in the culture.

In several instances, the A donor had been immunized intradermally with KLH (*6*); a blastogenic response to KLH was elicited in one-way MLC after the ^{3}H-T treatment, providing further evidence that a range of normal responses in vitro was retained by A cells after deletion of the response to transplantation antigens present on B_i leukocytes. Less complex studies of the KLH response were performed with cells from a single donor who had been immunized with KLH in vivo. The response to KLH in vitro was deleted with ^{3}H-T in culture; however, a response to PHA was still elicited, indicating that such selective ^{3}H-T deletions of response are not limited to allogeneic mixed cell responses and that they can also be obtained to soluble proteins. When standard MLC is carried out in the absence of the radioactive ^{3}H-T, 30 percent or more of the lymphocytes are proliferating when harvested at 5 to 7 days. Therefore, our observation that less than 1 percent of the lymphocytes in MLC are labeled in the course of ^{3}H-T treatment indicates that extensive clonal proliferation must normally occur before the reaction is detected by conventional techniques.

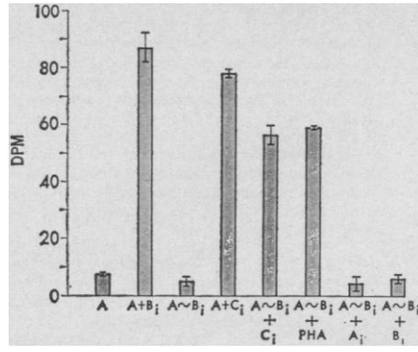

Fig. 2. Incorporation of [^{14}C]thymidine into DNA in MLC. A, B, and C refer to leukocyte populations from three unrelated and histoincompatible normal donors; ($_i$), cell population inactivated with mitomycin; (+), cells in the mixture; (~), mixed cells were treated with ^{3}H-T. Additions made after the ^{3}H-T was washed out are designated by (+) in the second line beneath the bars. Deletion of the response of A to B by treatment with ^{3}H-T did not prevent a response to C or to PHA. In this study, three donors were used whose HL-A phenotypes were known; there was no overlap in any factor. The lymphocyte stimulation results shown here are representative of more than ten similar studies of the ^{3}H-T treatment with different sets of donors. Reciprocal studies with these three specific donors showed that each donor responded in similar fashion when the other two were used as sources of the inactivated cell populations. *DPM*, disintegrations per minute.

Although we have yet to observe exceptions to the phenomenon reported here, it is possible that certain clones in a population might have a significantly longer generation time or might not have adequate initial contact with insoluble cellular antigens. In either case, cells undergoing a delayed response might not be destroyed within the time intervals discussed above. In the instance of a clone with a prolonged generation time, demonstration of reactivity might require significantly longer total culture periods than those used here.

Our present observations suggest several possibilities for further investigation in areas related to transplantation immunology. We believe that the treatment with ^{3}H-T may provide a means for inducing immunologic tolerance to transplantation antigens. Whether a population of cells made tolerant in vitro would retain the capability to mediate donor-type antibody production and cellular immunity after transplantation into a tolerant host is not yet known, but initial observations with the technique described here suggest that such modified cells may retain these immunologic potentials (*7*). If so, then at least two clinical ramifications related to immunologic deficiency syndromes and to cancer are apparent. While children with congenital deficiencies may be inherently tolerant, immunologic tolerance has also been attained in cancer patients after administration of cyclophosphamide in the manner of Santos *et al.* (*8*). The graft-versus-host syndrome has been a frequent and often lethal consequence of lymphoid grafting to tolerant recipients. As suggested previously, modification of cell populations in vitro might provide a means of eliminating this donor-mediated complication (*9*).

Since those clones of immunocompetent cells responding to histocompatibility antigens can be destroyed in vitro while the remaining cells retain their capability to respond to other cellular antigens, an adoptive immunotherapy for cancer exploiting this capability can be considered. With use of these techniques for induction of bidirectional tolerance to transplantation antigens of the donor (^{3}H-T MLC in vitro) and recipient (chemotherapy in vivo), a graft of a modified cell population might recognize tumor-specific antigens, proliferate, and destroy tumor cells without destruction of the tumor-bearing host.

Our observations not only support the theory that clonal selection is fundamental to the immune response, but also indicate that selective destruction of clones proliferating in response to transplantation antigens may prove useful for surmounting the histocompatibility barrier.

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References and Notes

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Uncoupling Cell Junctions in a Glandular Epithelium by Depolarizing Current

Abstract. *The high electrical conductance linking adjacent border cells in* Chironomus *salivary gland is depressed reversibly when current is passed outward from one of the cells, though not when current is passed inward. This "uncoupling" is closely associated with an electrically induced increase in conductance in the (nonjunctional) membrane of that cell.*

Several different treatments can depress the high electrical conductance that characterizes cell junctions in a wide variety of tissues (*1–6*). During a study of such cell "uncoupling" in the salivary gland of *Chironomus* by cooling and by certain metabolic inhibitors, we noted that the loss of electrical coupling was generally accompanied by a decrease in cell membrane potential (*6*). This prompted us to inquire whether a simple shift of membrane potential can uncouple these cells. In four kinds of nerve cell linkages the conductance of junctions is known to change transiently in response to electric fields or currents impressed between cell interior and exterior (*1, 2, 7, 8*). We now find that cell junctions in *Chironomus* salivary gland show a reversible decrease in conductance, with some distinctive features, during passage of outward current from a cell.

Salivary glands of mid-fourth-instar larvae of the midge *Chironomus thummi* are used. To test for junctional coupling, we pass a step of electric current between the inside of one of the large border cells and the outside; meanwhile we record the potentials in this cell (E_I) and in a contiguous one of the same type (E_{II}). Comparison of the net changes of potential (V_I and V_{II} in Fig. 1D) as a steady state is reached shows the degree of coupling (*9*). Details of the method appear elsewhere (*6*).

Figure 1A shows the effect of depolarizing (that is, outward) current steps of different sizes. With small currents, E_{II} follows E_I closely in time course and magnitude, giving the voltage response typical of a linear, time-invariant resistance-capacitance network. In displaying such good electrical coupling, the cells show that they are linked by junctions of high conductance (*10*). But when the currents exceed about 10^{-7} ampere, the coupling falls markedly after some delay, and remains depressed while the current continues. First, an inflection appears in the E_I trace; E_I shoots up to a peak, then subsides to a steady value substantially larger than the normal values seen with slightly smaller current. E_{II}, however, follows the rise of E_I only briefly, then declines to a steady level smaller than is seen with slightly smaller current. The greater the current, the earlier these events develop. The larger outward currents thus diminish V_{II} even though V_I increases. These features are seen consistently in fresh preparations (37 cases). The general result is brought out especially clearly in Fig. 1B, which gives the steady-state current-voltage characteristics for V_I and V_{II} with outward current. The failure of V_{II} to keep pace with V_I as the current is increased reflects a substantial fall in junctional conductance during the larger currents. The alternative possibility, that the membrane conductance of cell II increases substantially, is untenable since no such change is evidenced in the time constant of E_{II} at cutoff of a steady current (*11*).

The course of E_I during large depolarizations is not that of an action potential. As Fig. 1D shows, we find no response capable of sustaining itself once the driving current is stopped. Tests (not shown) with small 140-msec square pulses of current superimposed on the uncoupling current step confirm that the secondary rise of E_I reflects a a rising input resistance and the later decline, a partial subsidence of the input resistance of the cell system. Testing with a small current pulse, after cutoff of the large depolarizing current, reveals that normal coupling is restored within a few tenths of a second.

When applying hyperpolarizing current, we have seen no change in coupling (Fig. 1C), even with currents and potential shifts that would more than suffice to uncouple were they depolarizing. The steady-state hyperpolarizations have ranged up to the largest that the cells can sustain, about 100 mv negative from resting potential.

A pair of contiguous cells behaves symmetrically with respect to an outward current passed from either cell. This is evident from the experiment of Fig. 1C. Here a current lead and a voltage probe are present in each of the two cells. A depolarizing current of given size in either cell uncouples in roughly the same length of time. The symmetry seen here has been confirmed in each of three trials (*12*). Since a junction's susceptibility to uncoupling