tion (that is, $-\gamma/2 = 0$) the equation in Fig. 4 reduces to the familiar Langmuir adsorption isotherm.

The above experiments were repeated at three different concentrations of ouabain in the recovery medium. If the action of glycosides were mediated through the cooperative mechanism, it must be reflected by systematic shifts in $K_{K/Na}$ and $-\gamma/2$. The results have confirmed this prediction (Fig. 3). It is seen that the same cooperative adsorption isotherm can describe the equilibrium uptake of potassium at all concentrations of ouabain. The curve is, however, shifted to the right; that is, the selectivity is progressively lowered with increasing concentrations of ouabain. The values of the parameters used in these plots are summarized in Table 1. The empirical relationship between the intrinsic equlibrium constant and ouabain concentration (Fig. 5) was derived by means of log-log plot and standard regression techniques.

One explanation of the above findings is that the sites responsible for ion accumulation undergo changes of conformation in the presence of ouabain. The mechanism for inducing such changes is not known. It is attractive, however, to consider that ouabain may act on ion accumulation by binding noncompetitively to specialized sites (cardinal sites) (*2*) on cytoplasmic as well as membrane proteins. This could alter the pattern of electronic distribution (*11*) over an extended group of sites. This affects the field strength of the anionic adsorptive sites. On a theoretical basis it is known that the intrinsic equilibrium constant (the selectivity ratio for potassium over sodium) is controlled by the field strength of the anionic sites [(*2*); see also (*12*)].

The presence of more than one type of site for glycoside activity offers potential for the integration of different pharmacological effects. Indeed, the uptake of cardiac glycosides in red blood cells (*13*), unmyelinated nerve (*14*), and other mammalian cells (*6*) consists of at least two fractions: surface transport sites and nonspecific sites. Surface sites may control some aspects of cell function as exhibited by the inhibition of sodium efflux in the presence of external ouabain (*15*). A separate set of sites could explain the inotropic activity of ouabain injected into crab muscle fiber (*16*). The shifts of potassium accumulation in vascular smooth muscle also reflect that the site of cardiac glycoside action may be on cytoplasmic proteins. In conclusion, the above results indicate that the application of cooperative adsorption isotherm offers a quantitative basis for interpreting biological resetting induced by the action of drugs.

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$$\log \frac{K_{ad}}{F_T - K_{ad}} = n \log \frac{[K]_{ex}}{[Na]_{ex}} + n \log K_{K/Na}$$

This equation is the approximate form of Fig. 4 and holds when K_{ad} approaches half saturation ($F_T/2$). Therefore, once F_T is found from the experimental data, the value of n ($= e^{-\gamma/2RT}$) and $K_{K/Na}$ is fixed according to the above relationship; n is the slope of the straight line on log-log plot, and $K_{K/Na}$ is the inverse value of abscissa at ordinate value of unity.
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Specificity of Allogeneic Cell Recognition by Human Lymphocytes in vitro

Abstract. *Human lymphocytes proliferate in vitro in response to foreign histocompatibility antigens that are present on allogeneic lymphocytes. Within a population of immunocompetent lymphocytes there are specific subpopulations that respond to allogeneic cells from different individuals. A means of selectively eliminating such subpopulations is suggested.*

The major histocompatibility (H) systems in several species are complex in that each system is highly polymorphic with respect to alleles (haplotypes) and the number of different antigens associated with each system. Studying the graft-versus-host response in the chicken, in which lymphocytes respond to foreign H system antigens, Simonsen noted that up to 3 percent of lymphocytes respond to a single difference in H locus (*1*). A similarly high frequency of initially responding units in homograft reactions was subsequently found when the mixed leukocyte culture (MLC) technique (*2*) was used as a model of the recognition phase of the homograft or graft-versus-host reaction.

These two observations—the high frequency of initially responding units to single major differences in the H system where no overt sensitization had taken place, and the large number of antigens associated with each system—have led to the suggestion that the cells responding in this "primary" immune response are either extensively pluripotent or in fact totipotent. Alternatively, different cells may respond to different allogeneic antigens, and the high frequency of responding units can be explained in other ways (*1–3*). We have presented human MLC data consistent with this latter model. Stimulation in the MLC test—a measure of antigenic disparity at HL-A, which is the major H system

in man—is quantified by determining the incorporation of radioactive thymidine into lymphocytes responding to allogeneic, stimulating cells (*4*). Within families, sibling pairs differing by two HL-A haplotypes stimulate approximately twice as much on the average as those differing by only one allele (*5*). This observation has been confirmed in both man (*6*) and rat (*7*). While these results are consistent with the hypothesis that separate cell populations respond to different antigens on the two haplotypes—and thus there is an additive effect—other models involving threshold effects in totipotent cells are also possible. Further, the induction of tolerance in vivo in the rat, which results in specific nonreactivity in MLC (*7*), could be explained by the presence of either specific blocking factors (*8*) or specific receptor molecule modulation (*9*) in a totipotent responding cell model.

A more critical method of ascertaining whether cells show specificity—namely, that different populations of cells respond to different allogeneic cell stimuli—is to take advantage of the proliferative phase of the MLC response by eliminating or inactivating cells in culture that respond to an initial allogeneic cell stimulus. The incorporation of 5-bromodeoxyuridine (BUdR, a thymidine analog) into DNA of dividing cells with subsequent exposure to light in the visible or near-visible region inactivates responding cells. If the hypothesis of separate cell populations is valid, then, while such a treated population should no longer respond to that same stimulating cell, it should respond to different allogeneic stimuli. Using this approach we have shown such specificity exists for soluble antigens to which the donor of the cells is sensitized (*10*). We now present evidence that similar specificity exists in MLC responses.

Leukocytes were obtained from heparinized peripheral blood of healthy human donors by centrifugation (150*g* for 10 minutes) of the leukocyte-rich plasma (the portion remaining when the red cells had settled out in 2 to 3 hours). Mononuclear cells were separated from polymorphonuclear cells and red cells by the Ficoll-Hypaque gradient method (*11*). Stimulating cells were treated with mitomycin-C, suspended in tissue culture medium 199, containing Earle's base (Gibco) and supplemented with antibiotics and fresh cell-free plasma (*4*).

Table 1. Reciprocal restimulation with two allogeneic stimulating cells in human mixed leukocyte cultures after BUdR-light treatment. Results are expressed as the mean counts per minute of triplicate cultures ± standard error.

Initial MLC	BUdR	Secondary stimulant		Day of assay	Radioactivity (count/min ± S.E.)
		Type	10^6 cells		
AA_m	No	None		6	469 ± 104
AA_m	Yes	None		6	298 ± 104
AB_m	No	None		6	3802 ± 1089
AB_m	Yes	None		6	214 ± 1
AC_m	No	None		6	7329 ± 1834
AC_m	Yes	None		6	188 ± 6
AB_m	Yes	A_m	0.5	10	214 ± 21
		B_m	0.25	10	400 ± 126
		B_m	0.5	10	511 ± 75
		B_m	1.0	10	752 ± 343
		C_m	0.25	10	2715 ± 236
		C_m	0.5	10	2943 ± 640
		C_m	1.0	10	3160 ± 672
AC_m		A_m	0.5	10	414 ± 91
		B_m	0.25	10	1713 ± 497
		B_m	0.5	10	4263 ± 783
		B_m	1.0	10	5313 ± 232
		C_m	0.25	10	360 ± 73
		C_m	0.5	10	600 ± 167
		C_m	1.0	10	571 ± 60

Stimulating cells (2 to 6×10^7 cells) were mixed with responding cells (0.25 to 2.0×10^6 cells) in 8 ml in tissue culture dishes (15 by 60 mm) (Falcon Plastics). Equal doses of BUdR were added to appropriate dishes immediately after cell mixing and at 24-hour intervals for the first 96 hours of culture, to give a final BUdR concentration of $1 \times 10^{-5} M$. The cells were treated with fluorescent light (*10*), washed, and resuspended in tissue culture medium for subsequent use as responding cells. Fresh stimulating cells were added to an equal number of responding cells in wells of microtiter plates (Linbro) (*12*). Growth was measured on days 6 and 10 by measuring the uptake of tritiated thymidine into cells of replicate cultures (*4*).

The initial response of cells of individual A to the stimulating cells of B (AB_m) and C (AC_m) are given. In the second half of Table 1 the results of restimulation of the cultures after BUdR-light treatment are shown. When cells of A were initially stimulated with B_m, there was no subsequent restimulation with several different concentrations of B_m; restimulation with C_m results in a significant response at all three concentrations; alternatively, initial stimulation of A with C_m eliminates the later response to C_m but allows significant restimulation with B_m.

Two different experiments, both showing specificity, and also other phenomena are shown in Table 2. Initial stimulation with the allogeneic cells was significant in all cases, and this initial response was eliminated by treatment with BUdR and light. The results are those of restimulation after the initial stimulation followed by the BUdR-light treatment. In experiment 1, stimulation is obtained with both E_m and F_m after BUdR-light treatment of the control mixture DD_m. If the cells of D are first stimulated with E_m, the secondary response to E_m is eliminated whereas there is a response to F_m. Initial stimulation with F_m selectively eliminates the response to F_m leaving intact the response to E_m. In this experiment the response to F_m after the initial stimulation with E_m and the response to E_m after initial stimulation with F_m are comparable to response in the control situation where there was no initial allogeneic stimulation.

In some experiments we were unable to eliminate the secondary response to the initial stimulating cell and found a "potentiated" secondary response to a different allogeneic stimulating cell. In Table 2, experiment 2, there is a response to H_m after initial stimulation of G with H_m. This response, however, is lower than the response to H_m in the control situation (initial mixture GG_m). After initial stimulation of G with H_m the secondary response to I_m appears "potentiated" over the response to I_m in the control situation. After initial stimulation with H_m the secondary re-

Table 2. Reciprocal restimulation after BUdR-light treatment with both normal (experiment 1) and potentiated (experiment 2) responses to a different secondary allogeneic cell stimulus are shown. Secondary stimulating cells (1.0×10^6) were placed in each well. Results are expressed as mean counts per minute of triplicate cultures ± the standard error.

Initial MLC	Secondary stimulant	Radioactivity (count/min ± S.E.)
	Experiment 1	
DD_m	D_m	2259 ± 443
	E_m	12,836 ± 781
	F_m	5694 ± 328
DE_m	D_m	1872 ± 685
	E_m	1609 ± 78
	F_m	5371 ± 1775
DF_m	D_m	1852 ± 681
	E_m	8038 ± 1908
	F_m	1357 ± 259
	Experiment 2	
GG_m	G_m	202 ± 21
	H_m	7047 ± 1572
	I_m	14,649 ± 2527
GH_m	G_m	432 ± 43
	H_m	4310 ± 1975
	I_m	37,356 ± 1859
GI_m	G_m	277 ± 69
	H_m	17,646 ± 1244
	I_m	5276 ± 311

sponse to I_m is significantly greater than the response to H_m. The reverse situation holds true after initial stimulation of G with I_m, clearly demonstrating specificity.

We have done ten combinatorial experiments similar to those presented above in which reciprocal restimulation was seen; that is, the secondary allogeneic stimulating cell which was different from that used initially to activate the responding cells resulted in a greater response of the responding cells than when the secondary and primary stimulating cells were from the same individual. In two other combinatorial experiments we were unable to interpret our results. In one case there was no initial stimulation in the allogeneic mixtures. In the other case the "response" at 10 days with the secondary addition of "stimulating" cells isogeneic to the responding cells was in the range of 10×10^3 to 20×10^3 counts per minute. Neither of the allogeneic secondary stimulating cells produced a response above that of the control.

The simplest interpretation of our results is that treatment with BUdR-light kills the cells that specifically respond to alloantigens and leaves intact other cells that can respond to other alloantigens. Although our results do not differentiate between a unipotent and a multipotent model, they would rule out a totipotent cell model. Another possible interpretation of the results is that, in the MLC, responding lymphocytes produce a specific blocking factor directed at the antigens of the stimulating cells. This blocking factor could either cover foreign antigens on the stimulating cells or, by combining with the antigen on the stimulating cells, then act—as an antigen-antibody complex—to "tolerize" specific responding cells. Experiments designed to find such an inhibitory factor have been negative so far. For instance, there appears to be no selective inhibitory effect of a culture JK_m treated with BUdR and light on a freshly prepared mixed culture JK_m as compared to JL_m. Whereas more complicated explanations are possible, our experiments suggest that different cell populations do respond to different allogeneic cell stimuli.

Earlier we stressed the need to test MLC mixtures at several concentrations of stimulating cells to maximize the probability of seeing a response if one can occur (*5*). All experiments in our study were done with a range of cell concentrations.

Many facets of this reaction are still not understood. Primary among these is the potentiated response seen in some of the experiments of the secondary allogeneic stimulus after BUdR-light treatment of an initial allogeneic MLC. We have described a potentiating factor which can be produced in activated lymphocyte cultures (*13*), part of whose activity may be cell-bound and could thus explain our results. The observation of a significant secondary response to the same stimulating cell used to initially activate the responding cells could be explained similarly. Perhaps the cells capable of responding to the initial stimulus do in fact respond and are eliminated, but a potentiating factor allows other cells to respond.

There is specificity of the cellular immune response after sensitization, which has been demonstrated in vitro both for initial antigen recognition (*10*) and for target cell destruction (*14*). The question of cellular specificity in the "unsensitized" homograft recognition response could, however, not be answered by studying cellular specificity in sensitized systems.

The existence of different cell populations responding to different allogeneic cell stimului can be explained in several ways (*1–3*). It is still not possible to differentiate between two of the more likely explanations consistent with the high frequency of initially responding units: a pluripotent cell model or a high degree of cross-reactivity between the various major H system antigens as recognized by lymphocytes mediating cellular immunity.

There are practical implications of these studies. Our increased understanding of the recognition phase of the homograft reaction may help to achieve better donor-recipient pairing for transplantation. Further, a system that eliminates from an antigen-sensitive cell population the cells that can respond to allogeneic histocompatibility antigens in a potential recipient could be used to avoid the graft-versus-host reaction in transplants involving immunologically competent cells.

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