gens as assayed on day 11. Cell cultures that initially responded to SK-SD and underwent BUdR-light treatment, could no longer respond significantly to SK-SD but did respond to tetanus and mumps antigens. Similarly, cultures that initially responded to tetanus or mumps and underwent BUdR-light treatment could no longer respond to the homologous antigens but did respond to the heterologous antigen. In each case it is important to compare the response of the secondary stimulant with the response at day 10 of cultures receiving an initial stimulant but no secondary stimulant.

The experiment presented in Table 1, showing reciprocal restimulation in all combinations between three soluble antigens, must be considered as strong evidence that, at least in part, different cells are committed to respond to different antigens, that is, the cells are not totipotent. As seen from the day 7 assays, the cells responded to soluble antigens given initially, and BUdR effectively eliminated this response. Such cultures after BUdR treatment could no longer respond significantly to the homologous antigen added at several concentrations but did respond to heterologous antigens. In those cultures that could be restimulated with heterologous antigens the response was sometimes significantly higher or lower than the response when that antigen was added to initially unstimulated cultures but treated with BUdR and light. Although cross-reactivity between the antigens, pluripotentiality of cells, or cell-bound potentiating factors would explain these findings, other possibilities exist.

Stimulation of a culture by optimum or nearly optimum concentrations of antigen has consistently allowed restimulation by heterologous antigens or phytohemagglutinin after BUdR treatment. The optimum stimulating dose of a soluble antigen was determined by maximum incorporation of tritiated thymidine on day 7 of culture. We have made 23 experiments testing reciprocal restimulation with five different soluble antigens and several cell donors. In 18 cases the restimulation was reciprocal (that is, the response to the homologous antigen is completely eliminated in both directions, and the cultures could still respond to the heterologous antigen). In the other five cases, after the response to the homologous antigen was eliminated, there was restimulation with the heterologous antigen in one direction, but no restimulation in the other direction. In no case have we found a culture that, after initially responding to one antigen and undergoing inactivation with BUdR, could still respond to the homologous antigen but not to a heterologous antigen to which the individual was sensitive.

Our data suggest that cells responding to soluble antigens in leukocyte cultures have specificity. Although complicated models could be proposed to invalidate this conclusion, it would seem very unlikely that responding cells are totipotent. Our results do not allow us to differentiate between a unipotent cell model and a pluripotent one; however, they do suggest that there is specificity in the "thymic" dependent cell population.

Leukocyte cultures are stimulated by a variety of substances. The use of BUdR to specifically kill only those cells that are synthesizing DNA in response to a stimulus should have considerable application in determining if further functional subpopulations exist among peripheral blood lymphocytes. For instance, one can test whether the cells that respond to nonspecific stimuli (such as phytochemagglutinin and pokeweed mitogen) include those cells that respond to specific stimuli (soluble antigens). This method should also be useful in investigating more complex cross-reacting antigen systems like those in mixed leukocyte cultures. The frequency of the initial responding unit is quite high, with minimum estimates in the range of 1 of 200 to 1 of 50 cells initially responding to a single allogeneic cell stimulus (*6*). The frequency of the initial responding unit in cultures stimulated by soluble antigens is not known. It will be interesting to determine whether there remain cells in a culture which are capable of responding to a second allogeneic cell stimulus after all the cells responding to the initial allogeneic cell stimulus have been eliminated by BUdR treatment.

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Cytotoxicity: Specificity after in vitro Sensitization

Abstract. *Animals sensitized in vivo against an allogeneic tissue subsequently show accelerated rejection specifically of that or antigenically similar tissues. Lymphocytes sensitized in vitro will destroy target cells isogeneic with the sensitizing cells. Lymphocytes sensitized in vitro can differentiate specifically between different allogeneic target cells—as occurs in vivo.*

The homograft response can be studied with in vitro models such as the mixed leukocyte culture (MLC) system (*1*, *2*) in which lymphocytes respond to foreign histocompatibility antigens on allogeneic cells, and target cell destruction assays in which aggressor (sensitized) leukocytes destroy allogeneic tissue (*3*). Extrapolation from in vitro models to the in vivo situation requires convincing evidence of a significant parallelism. We now report on the homograft response, in particular the specificity of tissue destruction after in vivo or in vitro sensitization.

An individual sensitized in vivo will show accelerated rejection of tissues from the sensitizing individual or tissues sharing antigens with the sensitizing tissue. That same individual will not, however, reject all grafts in an accelerated fashion and will not destroy autologous tissue. Likewise, sensitized leukocytes from that individual will, in vitro, preferentially kill target cells isogeneic to the sensitizing cells (*4*). In contrast, several reports suggested that leukocytes activated in vitro with a variety of agents—such as phytohemagglutinin, antigens to which the donor of the lymphocytes is sensitized, or those in MLC (*5*)—appear to have

no specificity of killing; rather they are cytotoxic to any allogeneic cell as well as to isogeneic cells.

Ginsburg, Feldman, and their associates (*6*) demonstrated that rat lymphocytes "sensitized" on mouse cell monolayers were more cytotoxic to target cells isogeneic with the sensitizing cell. While not ruling out a nonspecific component of the reaction, they did demonstrate specificity. In fact, no such specificity is observed in vivo in xenogeneic combinations.

Mouse lymphocytes sensitized against allogeneic cells in MLC are cytotoxic to target cells (lymphoid cell lines) isogeneic to the sensitizing cells but not to target cells isogeneic to the sensitized cells (*7*). In these studies a test was not made to determine whether cells sensitized to one strain of allogeneic cells are more cytotoxic to target cells of that strain than to target cells of another allogeneic strain, a form of specificity seen after sensitization in vivo. The most dramatic aspect of in vivo specificity is that sensitized cells differentiate between different allogeneic cells.

Our study shows that in man there is specificity of destruction when tested on different allogeneic target cells after sensitization in vitro. Three lymphoblastoid cell lines established from the peripheral blood of different healthy unrelated donors were used as target cells; these same cells as well as normal peripheral blood leukocytes of other individuals were used as sensitizing cells.

The three lymphoblastoid cell lines (Associated Biomedic Systems) are designated A (HL-A 1,2,8), B (HL-A 2,7,Te54) and C (HL-A 3). For instance, responding cells of an individual X are sensitized in vitro by mixture with mitomycin C (subscript "m")—treated "sensitizing" cells of lymphoblastoid cell line A in mixed culture, XA_m. After 5 days of incubation, the cytotoxic potential of the mixture XA_m is tested in triplicate on ^{51}Cr-labeled target cells (*8*) of cell line A (the combination is designated XA_m/A) or against labeled target cells of B (XA_m/B). Combinatorial experiments were done: that is, XA_m/A was compared with XB_m/A, and XB_m/B was compared with XA_m/B. It was only possible to compare combinations involving the same target cell since there is a significant variation in the propensity that different lymphoblastoid cell lines have to release ^{51}Cr. It is necessary to do combinatorial experiments to rule out that one mixed culture such as XA_m is simply more active than XB_m and that this is the reason for the greater cytotoxicity in the combination XA_m/A than in XB_m/A.

Table 1. Cytotoxicity (percent) in a three-way combinatorial experiment. Values given are those obtained after 24 hours of incubation; LA_m/B was not tested.

MLC	Target cells		
	A	B	C
LA_m	29.1		17.5
LB_m	6.2	65.5	9.8
LC_m	5.5	26.3	46.6

"Specificity," as used in this paper is demonstrated if greater cytotoxicity exists when the sensitizing cells and the target cells are isogeneic than when they are allogeneic. The results indicate that after activation in vitro there is specificity. It is impossible, because of possible antigenic sharing or cross-reactivity between the various target cells, to decide whether there is a nonspecific component as well.

Mixed cultures were sensitized as described (*2*). Peripheral blood leukocytes were suspended in tissue culture medium 199 (Grand Island Biological) supplemented to contain penicillin (100 unit/ml), streptomycin (100 μg/ml), and 20 percent (by volume) of cell-free frozen or fresh human plasma (199-S:20). Responding cells were suspended to a final concentration of 0.75×10^6 mononuclear cells per milliliter. Cells to be used as stimulating (sensitizing) cells were treated with 25 μg of mitomycin C (subscript "m") per milliliter (Nutritional Biochemicals) for 20 minutes at 37°C, washed twice, and suspended at a final concentration of 2.5×10^3 to 6.0×10^5 leukocytes per milliliter. Lymphoblast cultures were maintained in suspension culture in medium RPMI 1640 in a humidified atmosphere containing 5 percent CO_2 at 37°C. Lymphoblasts to be used as stimulating cells were centrifuged at 150*g* for 6 minutes and suspended in 199-S:20 before mitomycin C treatment. Stimulating and responding cells (20 to 70 ml) were mixed and incubated in 250-ml glass erlenmeyer flasks in a humidified atmosphere containing 5 percent CO_2 at 37°C. On the 5th day of incubation the cultures were thoroughly mixed; two 1.0-ml portions were removed to glass culture tubes (16 by 100 mm) with metal closures and labeled with 2 μc of tritiated thymidine (specific activity 1.9 c/mole, Schwarz BioResearch) for 5 hours to assay the reaction in MLC as reported; the remaining cells were used as "agressor cells" in the cytotoxic assay.

Lymphoblasts for use as target cells were subcultured 3 to 5 days before use. The cells were centrifuged at 150*g* for 6 minutes and suspended in 199-S:15. Between 6×10^6 and 10^7 cells in 0.3 ml were mixed with 0.2 to 0.3 ml of Na_2CrO_4 (Amersham/Searle; approximately 200 to 300 μc of ^{51}Cr) in 40-ml plastic centrifuge tubes (Falcon) and incubated for 1 hour at 37°C in a humidified 5 percent CO_2 atmosphere with frequent agitation. Thereafter the cells were washed in cold phosphate-buffered saline containing 15 percent cell-free plasma. The washed cells were diluted to 0.04×10^6 cell/ml and kept on ice until combination with MLC cells. Viability of these cells assayed by trypan blue has varied between 93 and 99 percent.

To prepare the aggressor cells, the MLC cell suspensions were centrifuged at 150*g* for 6 minutes, washed once with 15 to 30 ml of 199-S:10, and diluted

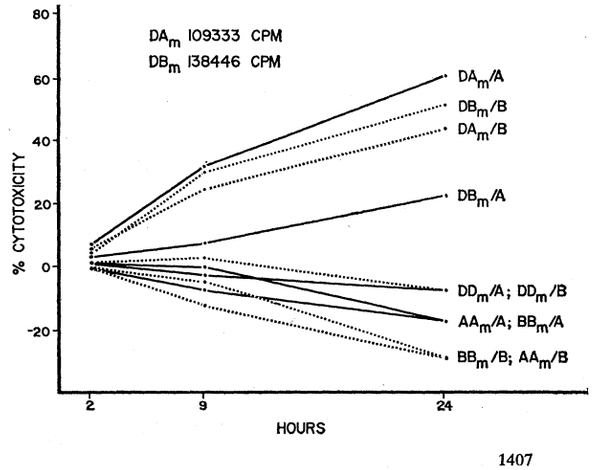

Fig. 1. Cytotoxicity results in various combinations. Solid lines represent combinations with A target cells; dotted lines, B target cells. Various control MLC such as AA_m and BB_m show no cytotoxicity despite extensive proliferation of A and B lymphoblastoid cells.

in 199-S:15 to 2 × 10^6 mononuclear cells per milliliter. Aggressor cell suspensions were distributed in 0.5-ml amounts to glass culture tubes (16 by 100 mm) with metal closures and warmed to 37°C. In the spontaneous release controls, 0.5 ml of 199-S:15 replaces the aggressor cell suspension. Target cells were brought to 37°C and added to aggressor cells in 0.5-ml amounts. Cell mixtures were incubated at 37°C in a humidified 5 percent CO_2 atmosphere, and triplicate cultures were removed for assay at 2, 6 to 9, and 24 hours. Release of ^{51}Cr was "stopped" by addition of 2.0 ml of cold phosphate-buffered saline which contained 15 percent cell-free plasma, and immediate centrifugation at 2000*g* for 5 minutes. Supernatants (1 ml) were placed in counting vials and release of radioactive chromium was assayed in a Packard γ-radiation counter. In each experiment the maximum release of radioactivity was determined by the addition of 2.0 ml of distilled water to triplicate target cell controls, which were subsequently frozen and thawed four times and centrifuged at 2000*g* for 10 minutes. The freeze-thaw supernatant radioactivity was arbitrarily equated to 100 percent (maximum) release.

The amount of radioactivity released spontaneously at each timed observation was subtracted from both the experimental and maximum release (freeze-thaw) values. The net experimental value was divided by the net maximum value to give the percentage cytotoxicity.

In a combinatorial experiment cells of an individual D were sensitized to cell lines "A" and "B" in mixed cultures, DA_m and DB_m. The cytotoxic potential of these mixtures was tested against the target cells of "A" and "B." Various control mixtures were also included. No discrimination was possible if the values at 2 hours were examined. The percentage cytotoxicity at 6 hours and at 24 hours, however, allowed a meaningful comparison and rank ordering in terms of percentage cytotoxicity. The combination DA_m/A showed a greater percentage cytotoxicity than DB_m/A; conversely, DB_m/B was greater than DA_m/B. While specificity thus exists in this case, the combination DA_m/B does show some degree of cytotoxicity, as does HB_m/A. An experiment testing all three cell lines in the various combinations (24-hour values of cytotoxicity) is given in Table 1. Specificity was again demonstrated.

We have done 12 experiments in which reciprocal combinations were included to test for specificity. In one experiment, specificity was shown in one direction but not in the other. In one other experiment, whereas the combination OB_m/B showed a greater percentage cytotoxicity than the combination OC_m/B, the two combinations OB_m/C and OC_m/C showed identical cytotoxicity. Whereas this last experiment might be used to support specificity to some extent, we have eliminated it from consideration and have based our analysis on the other 11 experiments.

The probability that two combinations tested reciprocally, as shown in Fig. 1, will show specificity in both directions by chance alone is 0.25. The probability, thus, of having specificity in both directions in 10 of 11 combinatorial exepriments by chance alone is $<.001$ (a conservative analysis since the various components of the combinatorial experiments are not considered individually)—strongly suggesting that after sensitization in vitro at least some component of the cytotoxic response is specific.

Additional experiments further support this conclusion. From the correlation between the degree of cytotoxicity and the extent of MLC activation [radioactive thymidine incorporated (count/min)], it appeared reasonable to compare two combinations such as XA_m/A and XB_m/A if the MLC's XA_m and XB_m incorporated approximately the same number of counts per minute. In 11 further experiments, in which reciprocity could not be tested, but in which the various cell mixtures were activated to the same extent, one-way specificity was demonstrated in every case, that is, the isogeneic combination showed a greater percentage of cytotoxicity than the allogeneic combination ($P < .0005$).

Certain aspects of these studies which are either not yet understood or have not been tested deserve consideration. First, in some combinations, such as DD_m/B and BB_m/B in Fig. 1, there is less ^{51}Cr released than in the labeled cells of B incubated alone. Further, the amount of this decrease is variable. In the combinatorial experiments reported, this cannot of course explain the specificity; in the other experiments, examination of the data shows no correlation between this effect and the specificity noted. Second, although in most of the cases in which different combinations were compared the differences were statistically significant, in a few cases this was not true (although on a simple ranking basis specificity was demonstrated). The nonparametric statistical evaluation of the data presented in this paper is not, however, dependent on the statistical significance of differences between the various combinations compared to test for specificity and the conclusions are thus valid at the *P* values given. Third, since lymphoblastoid cell lines were used as target cells and normal peripheral blood cells from the donors of the lymphoblastoid cell lines were not available to us, we could not test for the cytotoxic effect of a mixture such as AC_m (with the A cells being normal peripheral blood leukocytes) on lymphoblastoid target cells of A. Whereas this does not allow us to answer the question of possible nonspecific component of destruction, it again does not affect our conclusions of specificity.

The cytotoxicity in a combination such as DA_m/B (Fig. 1) may represent a nonspecific component of destruction, which may also exist in vivo (*9*) or be due to antigenic cross-reactivity between the cell lines "A" and "B." These cell lines in addition to sharing HL-A antigens may share a "blast cell" antigen. Cell-free media taken from the mixed cultures at 5 days were not cytotoxic in our assay.

These studies demonstrate, within allogeneic combinations in man, that after sensitization in vitro there is specificity of cytotoxicity. We would suggest, as have others, that the most likely explanation for the specificity is that the responding cells in the sensitizing cultures have specific receptor sites for antigens on the sensitizing, and therefore target, cells and that this is the basis of the specificity. It may be that when cells activated in vitro have no specific target in the test situation, they do mediate nonspecific cytotoxicity as discussed earlier. Whether a nonspecific component also exists, and to what extent, when the activated cells have a specific target will require further experimentation. These methods apparently may be used as in vitro counterparts of the in vivo homograft reaction.

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Postembryonic Development of Adult Motor Patterns in Crickets: A Neural Analysis

Abstract. *Adult crickets have stereotyped patterns of motor output which are generated by the central nervous system, and which serve as a standard against which emerging nymphal patterns can be measured. The neural circuits generating these patterns are not functional at hatching. The pattern elements appear in an ordered sequence over the course of the last four molts. The circuits are completely functional before the final molt. Circuits which might be prematurely activated are suppressed in the nymph by descending inhibition from the brain.*

Invertebrate behavior often reflects stereotyped patterns of motor neuron firing, called motor programs. The programs are generated by small groups of central neurons which can operate without phasic sensory feedback. Patterns of this type underlie locust flight, cricket stridulation, swimmeret beating in crayfish, and swimming and siphon withdrawal in mollusks (*1*). These patterns are so highly stereotyped in an individual, or between conspecifics, that they provide an excellent standard against which the activity of immature animals can be measured. In the case of stridulation, and presumably in all such programs, the patterns are not learned; the neural circuits generating them must be constructed during the course of development on the basis of genetically stored information. We have examined the appearance of two such motor programs during postembryonic growth, and have found that the circuits are not functional at the time of hatching from the egg, that elements of the pattern emerge in an ordered sequence over the course of the latter half of the nymphal life-span, that the circuits are complete before the final molt, and that completed circuits which might be accidentally activated are suppressed by descending inhibition.

The behavior patterns investigated were song production (stridulation) and flight in the field cricket *Teleogryllus commodus*. Both are exclusively adult behaviors, dependent upon the presence of the wings, which do not appear until the final molt. Furthermore, nymphs do not attempt to move their small wing pads (*2*), which are clearly visible, in the pattern appropriate for either behavior. This is particularly interesting in the case of aggressive stridulation because the nymphs do display other behaviors associated with aggressive interactions. Stereotyped motor programs underlie both behaviors; this was demonstrated first for locust flight, and later confirmed for cricket stridulation and flight (*3*).

We characterized nymphal behavior by recording action potentials with fine wires (20 μm) placed in identified muscles. Since the muscles are neurogenic (each nerve impulse produces a single muscle action potential), and since they are innervated by only one to three neurons, this procedure provides a precise monitor of identified motor neuron activity. The motor pattern of flight was elicited by activating the tarsal reflex (loss of contact with the substrate), and suspending the animal in a wind stream. Song patterns were released by making heat lesions in inhibitory areas (mushroom bodies) of the brain (*4*).

The crickets develop gradually into the adult form after nine, ten, or eleven molts (twelve stages including adult). Most individuals reach adulthood after ten molts. The number of molts an animal has completed can be determined by painting it, and repainting after each molt or, in a carefully controlled colony, by measuring skeletal size (we have done both). The structure of the wing pads, in late instar nymphs, indicates how many molts the animal will undergo before becoming an adult. In the case of song production, we recorded only from last instars; in the case of flight, we went as far back in development as any features of the pattern appeared (about ten animals were examined at each stage).

The acoustical repertoire of *T. commodus* is large, and the songs, although stereotyped, are complex (*5*). These insects use a variety of types of sound pulses, intervals between pulses, and numbers of pulses in a chirp. Last instar nymphs are able to generate nearly perfect motor patterns for the aggressive song, the courtship song, and the most highly structured part of one of the calling songs (Fig. 1). The last consists of five to seven loud pulses, with long intervals between the pulses, switching abruptly into a series of six to ten soft pulses with short intervals between pulses. The motor pattern produced by the nymph can be identified

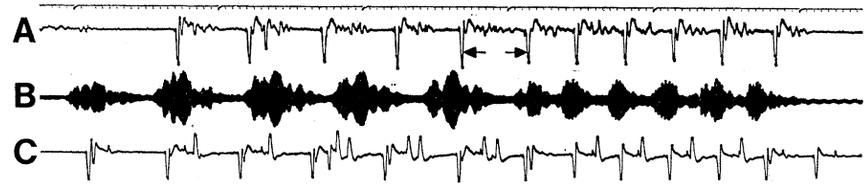

Fig. 1. Comparison of motor output of a nymph with the actual song of an adult. (A and C) Muscle action potentials recorded from a last instar nymph. (B) Sound pulses recorded from an adult (see text). (A) Second basalar muscle (wing opener); note how closely the number and spacing of nymphal muscle potentials correspond to the adult pattern. (C) Push-pull recording from subalar muscle (downward spikes, wing opener), and remotor muscle (upward spikes, wing closer); this demonstrates reciprocal firing of antagonists (closing movement produces sound pulse). Arrows indicate the switch (long intervals to left; short intervals to right), in both adult and nymphal patterns, from long to short intervals between pulses. Time calibration: 5 msec per small division.