Kinetic Differences in Unresponsiveness of Thymus and Bone Marrow Cells

Abstract. *Both thymus and bone marrow cells of adult mice can be made specifically unresponsive to human gamma globulin, but each cell population displays a distinct kinetic pattern for both the induction and spontaneous loss of the unresponsive state. These kinetics appear to be much slower in the bone marrow cells than in the thymus cells. In addition, the dose of deaggregated human gamma globulin needed to induce unresponsiveness in bone marrow cells is much greater than that needed to induce unresponsiveness in thymus cells. Apparently, unresponsiveness in only one cell type is sufficient for the tolerant state to be exhibited by the intact animal.*

Investigations of the cellular events manifested in the induction process of the state of immunological unresponsiveness have been limited to studies of whole animals or heterogeneous lymphoid cell populations, such as those obtained from the spleen or from the peripheral blood (*1*). These in vivo studies have indicated that this induction period is short, from 24 hours to 4 or 5 days depending on the antigen and the test system used. In view of the collaboration between thymus cells and bone marrow cells in the immune response to a number of different antigens (*2*) and the subsequent demonstration that unresponsiveness can exist at each of these cellular sites (*3*), cellular events in the induction of unresponsiveness now demand new study. We now report that in adult mice there exists a distinct kinetic pattern for both the thymus and the bone marrow cell populations with regard to both the induction and spontaneous loss of the unresponsive state to human gamma globulin (HGG). In addition, our data demonstrate differential susceptibility of thymus cells and bone marrow cells to varying doses of tolerogen (a physical form of antigen that induces tolerance).

Synergism has been observed in the response to HGG when both thymus and bone marrow cells were obtained from normal mice, but not if either cell type came from tolerant mice (*3*). From these data, the prediction can be made that unresponsiveness in one cell type would be sufficient to make an intact animal appear tolerant. To test whether such a cellular dichotomy exists, the kinetics of the induction and spontaneous loss of immunological unresponsiveness to HGG were determined separately for thymus cells and for bone marrow cells.

Groups of adult A/J male mice (*4*) were injected with 2.5 mg of HGG deaggregated by ultracentrifugation (*5*) and each group was killed at a specified time. Suspensions of their thymus cells (90×10^6) or bone marrow cells (30×10^6) were injected intravenously along with normal bone marrow cells (30×10^6) or thymus cells (90×10^6), respectively, into lethally irradiated recipients (*3*). At the same time and again 10 days later, these irradiated mice were challenged with 0.4 mg of aggregated HGG (*6*) and, 5 days after the second injection, their spleens were assayed for plaque-forming cells (PFC) to HGG (*7*). Since very few, if any, direct PFC were found by this system, the data are expressed only as indirect PFC detected with the use of goat antiserum to mouse gamma globulin at a concentration shown to be optimal.

The percentage of suppression of the response obtained in the recipients receiving one cell type from donors injected with tolerogen (deaggregated HGG) and one cell type from normal donors compared to the response obtained in animals receiving both normal cell types was then calculated (*8*) and plotted as shown in Fig. 1. Thymus cells from tolerogen treated animals were essentially unresponsive by day 2 and remained so for the duration of the experiment (day 49). In contrast, bone marrow cells from the same tolerogen treated donors did not begin to show unresponsiveness until day 11 and were totally unresponsive only after 21 days. In addition, by day 49 bone marrow cells had returned to the responsive state. The induction of unresponsiveness in the intact animal paralleled the induction of unresponsiveness in thymus cells. Thus, the kinetics of the induction of unresponsiveness with this experimental protocol are much faster in thymus cells than those seen in bone marrow cells, and the state of unresponsiveness persists much longer in thymus cells. It is also of interest that an animal with an unresponsive thymus cell population may appear tolerant, although it can possess bone marrow not yet rendered unresponsive (for example, day 5) or having recovered from unresponsiveness (for example, day 49). These data could account for the fact that Taylor (*9*) was unable to demonstrate specific unresponsiveness in bone marrow cells 24 hours after he administered a tolerogenic regimen of bovine serum albumin (BSA).

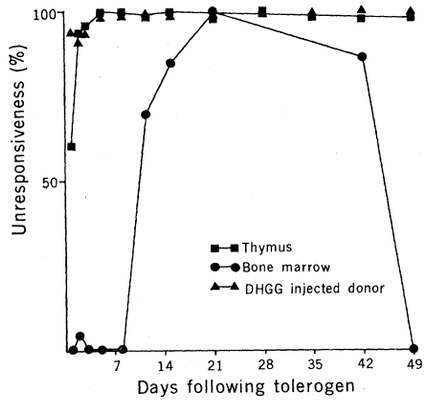

Fig. 1. Kinetics of the induction and spontaneous loss of unresponsiveness in thymus and bone marrow cells. Calculations of the values obtained for unresponsiveness in both cell types are described in (*8*); *DHGG*, deaggregated human gamma globulin.

To determine the effect of varying the dose of tolerogen on the induction of unresponsiveness in thymus and bone marrow cells, mice were injected with 0.1, 0.5, or 2.5 mg of deaggregated HGG. Eleven days later, cell suspensions of their thymus (90×10^6) or bone marrow (30×10^6) were injected along with normal bone marrow (30×10^6) or thymus (90×10^6) cells, respectively, into irradiated recipients. As before, mice were challenged with aggregated HGG on the day of transfer and again 10 days later, and their spleens were assayed for PFC 5 days after the second injection. The results of this experiment show that thymus cells obtained from each tolerogen treated group of mice were equally unresponsive (Fig. 2) although bone marrow cells obtained from the same tolerogen treated donors—that is, injected with 0.1, 0.5, or 2.5 mg of deaggregated HGG—were 9, 56, and 70 percent unresponsive, respectively. In summary, bone marrow cells require higher doses of tolerogen for the induction of unresponsiveness, they require a

longer induction period, and in these cells the unresponsive state is of shorter duration than that in thymus cells.

The distinct kinetic pattern for the induction of unresponsiveness in each cell population probably reflects a difference in the mechanism by which the unresponsive state is achieved. It may be that qualitative differences exist to account for the temporal differences seen in the kinetic data. While thymus cells may be rendered unresponsive simply by the passive interaction of tolerogen with cell, bone marrow cells may require an active process, such as cell division, differentiation, or antibody formation, to reach a tolerogen-sensitive stage. On the other hand, the kinetic differences might be quantitative so that more tolerogen is needed to confer unresponsiveness in the bone marrow population compared to the thymus population because either more antigen reactive cells are involved or more tolerogen per antigen reactive cell is required. By the same logic, the maintenance of unresponsiveness in bone marrow would necessitate continued high doses of tolerogen. When the dose falls below the threshold of this effective concentration, the bone marrow would be expected to return to a normal state, whether this recovery involves the derepression of unresponsive cells or the generation of new antigen reactive cells. The data on dose variation presented above (Fig. 2) and some recent findings by Mitchison (*10*) are compatible with such a hypothesis, as is the observation that there is a paucity of receptor sites on thymus as compared to bone marrow cells (*11*).

These data offer an insight into the cellular mechanisms operating both in the termination of induced immunological unresponsiveness (*12*) and in experimental autoimmunity (the termination of natural unresponsiveness) in rabbits (*13*). Each of these unresponsive states could be terminated by injection with either cross-reacting antigens or altered tolerated antigen, indicating that these tolerant animals still had the capacity to produce antibody specific for the tolerated antigen but lacked the ability to recognize the antigen as an immunogenic molecule. The observation would be explained by the involvement of two cell types: (i) the thymus cell which recognizes the immunogenic cross-reacting molecule and (ii) the bone marrow cell which is responsive to the tolerated antigen and which produces antibody. In view of

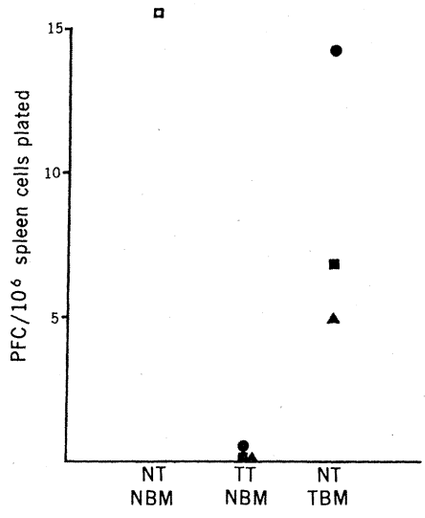

Fig. 2. The effect of tolerogen dose on the unresponsiveness of thymus and bone marrow cells. Each point represents the arithmetic mean of the individual response (PFC) obtained in six mice: NT, NBM; thymus, bone marrow obtained from normal donors. TT, TBM; thymus, bone marrow obtained from tolerogen injected donors which received 0.1 mg ●, 0.5 mg ■, or 2.5 mg ▲ of deaggregated human gamma globulin. □ Both thymus and bone marrow cells were from normal donors.

the results from the studies presented above, rabbits in which unresponsiveness to a heterologous serum protein had been induced at birth would be expected to contain at the time of testing (90 days after treatment with tolerogen) thymus cells that were unresponsive but bone marrow cells that had recovered from the unresponsive state. However, since thymus cells containing receptors for cross-reacting antigens would be present in the unresponsive animals, interaction could occur between these thymus cells (via determinants specific to the terminating antigen) and the bone marrow cells (via determinants cross-reacting with the tolerated antigen) resulting in a normal response to such cross-reacting determinants. The stimulation of bone marrow cells in this manner would result in their proliferation, differentiation, and subsequent synthesis of antibody with a specificity analogous to the receptor sites on the cells. It is known that in the case of natural unresponsiveness to thyroglobulin there are low levels of circulating antigen (*14*) which would, in a situation analogous to the dose response described above, be sufficient to render the thymus cells, and thus the entire animal, unresponsive to thyroglobulin but not sufficient to render bone marrow cells unresponsive. In accord with this hypothesis, cross-reacting or chemically altered thyroglobulins could be recognized as immunogenic by thymus cells and could stimulate the bone marrow cells to produce antibody specific for native thyroglobulin. These observations are compatible with the suggestion that thymus and bone marrow cells each may react with different determinants on an antigen (*15*).

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→ J. M. Chiller, G. S. Habicht, W. O. Weigle, *Proc. Nat. Acad. Sci. U.S.* **65**, 551 (1970). In this publication the possibility that tolerogen carry-over could induce unresponsiveness in transferred normal cells was judged unlikely by the inability to detect antigen on thymus or bone marrow cells from tolerogen treated mice. Subsequent experimental data substantiates this conclusion. Irradiated recipients of normal thymus, normal bone marrow, and either bone marrow, thymus, or spleen cells obtained from animals previously treated with tolerogen were capable of responding to aggregated HGG.
4. Purchased from the Jackson Laboratories, Bar Harbor, Maine.
5. Human γ-globulin (Cohn fraction II) was obtained by courtesy of the American Red Cross and further purified by chromatography on DEAE-cellulose at 0.01*M* phosphate buffer, *p*H 8.0. The desired fractions were pooled, concentrated, and diluted to a concentration of 30 mg/ml with 0.15*M* NaCl. The protein was then deaggregated by ultracentrifugation, 100,000*g* for 150 minutes in a swinging bucket rotor. The HGG contained in the top one-third of each tube was removed and diluted with 0.15*M* NaCl to 2.5 mg/ml. Protein concentration was estimated spectrophotometrically by absorbance at 280 nm ($E_{1\ cm}^{1\%} = 15$). Mice to be made unresponsive were injected intraperitoneally with 2.5 mg of the deaggregated material in a volume of 1 ml.
6. A solution of DEAE-purified HGG, 20 mg/ml in 0.01*M* sodium phosphate buffer, *p*H 8.0, was precipitated by heating at 63°C for 25 minutes, with occasional stirring, the mixture was then placed at 0°C for 12 hours. Enough 2.18*M* Na_2SO_4 was then added to reach a final concentration of 0.62*M*, and the mixture was incubated at 0°C for 30 minutes. The precipitate was washed three times with 0.62*M* Na_2SO_4, then dissolved in phosphate buffered saline, *p*H 8.0 (PBS), and dialyzed free of Na_2SO_4 against excess PBS. Protein nitrogen concentration of the aggregated solution was determined by micro-Kjeldahl analysis. The suspension was then diluted with 0.15*M* NaCl, *p*H 7.0, to contain 2 mg of protein per milliliter and stored frozen until used.
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8. At each time interval, individual spleens of the following groups of mice were tested for PFC. Recipients of (i) tolerant thymus (TT)

and normal bone marrow (NBM), (ii) normal thymus (NT) and tolerant bone marrow (TBM), and (iii) normal thymus (NT) and normal bone marrow (NBM). The size of each group at individual points varied from four to eight mice. The percentage of suppression in thymus cells or bone marrow cells from tolerogen treated donors was calculated at each point of the curve in the following manner:

$$\% \text{ suppression} = 100 - \left[\frac{\frac{\Sigma \text{ PFC in TT, NBM (or NT, TBM)}}{\text{No. mice treated}}}{\frac{\Sigma \text{ PFC in NT, NBM}}{\text{No. mice treated}}} \times 100 \right]$$

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Sex Attractant in a Brown Alga: Chemical Structure

Abstract. *The male-attracting substance produced by the female gametes of the marine brown alga* Ectocarpus siliculosus *was identified as allo-*cis*-1-(cyclohepta-dien-2′,5′-yl)-butene-1.*

Cellular chemotaxis is widely distributed in lower plants. Many cases of interaction on the gamete level are known, but so far there is only one system, the active compound of which is known; this compound is sirenine, which is produced by the female gametes of the water mold *Allomyces* (*1*). We shall report on the identification of the substance which is secreted by the female gametes of the marine brown alga *Ectocarpus siliculosus* (Dillw.) Lyngb. as an attractant for the male gametes. The sexual reaction in this species has been observed by many workers. Motile gametes are isomorphic. Female gametes, when settled on a surface, attract numerous male gametes which become attached to the female cell with the tip of their front flagellum. After one male gamete has fused with the female, the zygote loses its attraction for the other male gametes and they disperse. At the time that the attractant was first detected and isolated (*2*) only microgram quantities could be isolated by gas chromatography and assayed for its biological activity. By increasing the capacity of the cultivation apparatus, we have now obtained sufficient quantities of the gamone to identify its chemical structure.

For the production of the gamone, the female gametophytic clone D-A2 was used and grown in large quantities, essentially by the procedure described in (*2*). Between June 1968 and August 1970, 14,900 culture dishes were inoculated and harvested. The total harvest during this time was 1041 g of fresh gametophyte material, which corresponds to 154 g dry weight. Because of its high volatility, the gamone could be removed from the cultures by means of a stream of purified air, and condensed in a stainless steel trap at −80°C. This condensate was then flushed with a stream of N_2 through a drying tube containing $CaCl_2$ and into a glass trap at −78°C; the dry condensate was then dissolved in CCl_4. This procedure yielded a starting material of already good purity. The few contaminant compounds were then removed by preparative gas chromatography (Fig. 1A). The resulting pure gamone fraction was used for the analytical procedures. Quantitative assay of the gamone was made by gas chromatographic comparison with *n*-nonanal (*2*). During the 2 years of mass production, 92 mg of gamone were obtained. Our results indicate that the female gamone of *Ectocarpus siliculosus* is allo-*cis*-1-cycloheptadien-2′,5′-yl)-butene-1. This conclusion is based on the following evidence.

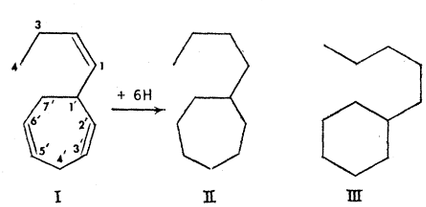
Table 1. Kovats indices of hexahydrogamone and authentic hydrocarbons for comparison. Columns used were: (A) 3.8 percent UCW 98 (methyl vinyl silicone gum); (B) 10 percent OV 225 (cyano silicone gum); (C) 2.5 percent Apiezon L; and (D) Apiezon L plus 2 percent Igepal.

Column	Temperature (°C)	Kovats indices		
		Hexahydrogamone	Amylcyclohexane	Butylcycloheptane
A	100	1165.14	1136.20	1165.01
B	60	1210.98	1166.88	1210.76
C	100	1191.24	1167.80	1191.15
D	172	1216.83	1176.43	1216.72

Mass spectrometry (Fig. 1B) shows that the mass of M^+ is 148. Comparison of the intensities of the masses 148 and 149 gives values between 10.2 and 11.6 for the number of carbon atoms per molecule. This means that the empiric formula is $C_{11}H_{16}$. The peaks at mass-to-charge ratios (m/e) of 133, 119, 105, and 91 indicate the successive loss of C_1 fragments. Peak 91 possibly represents the tropylium ion. The peak at m/e 146 arises from dehydrogenation in the inlet system. It is absent if the sample is introduced directly into the ion source. Elementary analysis suggests a hydrocarbon of the composition $C_{11}H_{16}$, and thus confirms the mass spectrometric data.

Proton magnetic resonance spectroscopy (Fig. 1C) confirms the presence of 16 protons per molecule. The following structural details are evident: one methyl triplet with chemical shift (τ) equal to 9.0 ppm, one tertiary proton (τ =6.6 ppm), six olefinic protons (τ = 4.4 to 4.8 ppm), two methylene protons between two double bonds (τ = 7.2 ppm, **VI**), and four methylene protons (τ = 7.9 to 8.0 ppm, **VII**). These data imply that the four unsaturation equivalents corresponding to the empiric formula $C_{11}H_{16}$ are represented by three double bonds and one ring system. The tertiary proton occupies the branched carbon atom holding the side chain.

The infrared spectrum (ultramicrocell, 50 μm path length, microilluminator) confirms the absence of functional groups. There is no band around 970 cm^{-1}, which means that there is no *trans* configuration at any of the double bonds. Two bands of medium intensity at 730 and 690 cm^{-1} indicate



I II III

$CH_3-C{=}C-$ **IV**

$CH_3-C-C{=}C-$ **V**

$=C-CH_2-C=$ **VI**

$-CH_2-C{=}C-$ **VII**