

## THE RESPONSE OF T CELLS TO HISTOCOMPATIBILITY-2 ANTIGENS

DOSE-RESPONSE KINETICS\*

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When sufficient numbers of immunocompetent cells are introduced into an animal that is incapable of rejecting them, a complex syndrome called graft-*versus*-host (GVH)<sup>1</sup> disease is produced (see references 1 and 2 for reviews of this subject).

The cellular events leading to this syndrome appear to be quite as complex as the syndrome itself. The grafted cells, after recognizing host antigens, divide and also cause host cells to divide (3–11). Recent evidence has indicated that at least two different types of graft cells, both of which have been thymus influenced (T cells), interact in disease production (12). Other recent evidence has suggested that antagonistic (antergistic) as well as synergistic cell interactions are involved (13).

A number of parameters have been used to quantitate the severity of GVH disease, the most common being the degree of splenomegaly produced in the hosts (1, 2). All of these parameters, however, are influenced to some degree by the interaction of the grafted and the host cells. In order to understand better the nature of this interaction, as well as possible interactions between the grafted cells, it would be desirable to measure the response of the grafted cells directly. Recent reports have shown that T cells (the cells which are responsible for the initiation of GVH reactions [see reference 2]) synthesize DNA in response to confrontation with the major histocompatibility antigens in lethally irradiated mice (14, 15).

We report herein a detailed analysis of dose-response kinetics in this reaction. Our results suggest that the kinetics usually show a suppressed first-order relationship, i.e., increasing the number of reacting cells by a given multiple increases the DNA synthesis by a fraction of that multiple. Occasionally either zero-order or higher order relationships obtain. These results are best explained by assuming the presence of both positive and negative interactions between T cells.

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<sup>1</sup> *Abbreviations used in this paper:* B, bone marrow influenced; GVH, graft-*versus*-host; IUDR, 5-iodo-2-deoxyuridine; L.N., lymph node; Sp., spleen; T, thymus influenced.

### *Materials and Methods*

*Production of GVH Disease.*—GVH reactions were produced in the offspring of C3H (H-2K)  $\times$  DBA/2 (H-2D) matings (CDF<sub>1</sub>) by the intravenous inoculation of C3H thymocytes. Recipient mice were lethally irradiated on the day of thymocyte inoculation.

*Mice.*—All mice were males from the Jackson Laboratory, Bar Harbor, Maine. After receipt, they were rested 1 wk in our colony before use. Recipients (CDF<sub>1</sub>) were 7–8 wk of age and thymocyte donors (C3H) were 5 wk old.

*Irradiation.*—Recipient mice received 900 R of X-irradiation from a Siemens 250 kv machine (Siemens Corp., Iselin, N.J.) with a 2 mm aluminum filter at a dose rate of 85 R/min.

*Cell Suspensions.*—Thymus cell suspension were made by gently squeezing thymuses between two sterile glass slides. The cells were then filtered through gauze and washed twice in cold, sterile medium 199 with 100 units/ml penicillin, streptomycin, kanamycin, and 10 units/ml heparin. Viable cells were counted using the trypan blue dye exclusion method.

*Measurement of DNA Synthesis.*—Uptake of the thymidine analogue 5-iodo-2-deoxyuridine (IUDR) labeled with <sup>125</sup>I was used as a measure of DNA synthesis. It has been shown that 24 hr after its inoculation, all IUDR not intimately associated with DNA is cleared from the lymphoid tissue (16). Thus, test mice were injected intraperitoneally with 2  $\mu$ Ci of <sup>125</sup>IUDR in a 0.2 ml volume 2, 3, 4, and 5 days after GVH inception, and their spleens and femoral lymph nodes were harvested 24 hr later. The <sup>125</sup>IUDR used in these experiments came from three different sources, Amersham-Searle Corp. (Arlington Hgts., Ill.), Schwarz Bio Research Inc. (Orangeburg, N.Y.), and New England Nuclear Corp. (Boston, Mass.), and varied between 2 and 6  $\mu$ Ci/ $\mu$ g in specific activity. At the time of inoculation of the IUDR into the mice, 0.2 ml of the isotope was placed in a counting tube. This served as a standard and after counting for 6 min in a Nuclear-Chicago (Nuclear-Chicago Corp., Des Plaines, Ill.) gamma counter, the standard minus the background was divided into the results minus the background; the results are therefore expressed as per cent uptake of label.

*Background DNA Synthesis.*—Previous work had shown that lethally irradiated mice incorporate very low levels of <sup>125</sup>IUDR during the first 5 days after irradiation (17). Thus, controls not inoculated with thymocytes were not routinely included in all experiments. Several of the experiments reported below did, however, include such controls. Also, since previous work had shown a return of DNA synthesis in the spleens of lethally irradiated mice on day 6 postirradiation (17), we confined our measurements to the first 5 days.

*Analysis of Relationship of DNA Synthesis to Cell Dose.*—One may assume, given an excess of all necessary reaction products, that increasing the number of inoculated cells by a given multiple should increase the amount of DNA they synthesize by that multiple. A deviation from this would imply that the reaction of one cell was affecting the reaction of another. In our analyses of this phenomenon we have borrowed the chemists' terminology in the following manner.

*Zero-order reaction:* DNA synthesis = (K) (number of cells)<sup>0</sup>; i.e., the uptake will be independent of the number of cells present.

*First-order reaction:* DNA synthesis = (K) (number of cells)<sup>1</sup>; i.e., a direct linear relationship between the uptake of IUDR and the number of cells should exist (triple the number of cells and triple the uptake).

*Second-order reaction:* DNA synthesis = (K) (number of cells)<sup>2</sup>; i.e., the uptake is dependent upon a two-cell interaction and thus is proportional to the second power of the number of cells present (triple the number of cells and nine times the uptake).

*nth-Order reaction:* DNA synthesis = (K) (number of cells)<sup>n</sup>; i.e., the reaction is dependent upon the interaction of *n* cells and thus its frequency is proportional to the *n*th power of the number of cells.

## RESULTS

In Fig. 1 we have plotted the uptake of label by parental thymocytes in the spleens and lymph nodes of lethally irradiated  $F_1$  recipients against time. Each point is the mean of determinations from three to four mice. The results come from 19 separate studies in 10 consecutive experiments with a variety of cell doses. Inspection of the data reveals the following trends. In the spleens, there

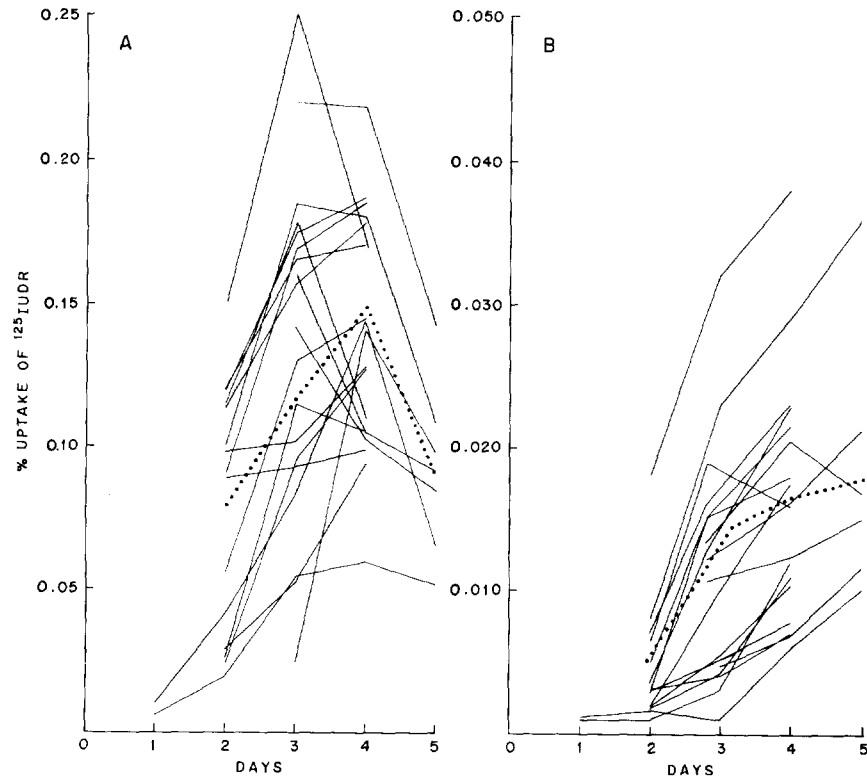


FIG. 1. DNA synthesis of different doses of parental thymocytes in the spleens (Fig. 1 A) and lymph nodes (Fig. 1 B) of lethally irradiated  $F_1$  mice, on various days after inoculation. The dotted line is the mean response.

was a tendency for a steep rise in DNA synthesis between days 2 and 3, followed by either a marked flattening of the curve or often a precipitous decline. In those cases where there was an increased uptake between day 3 and day 4, which occurred only in low responders, a sharp decline occurred on day 5. In the lymph nodes, on the other hand, there was a delay before the inception of significant DNA synthesis and a concomitant delay or even absence of the falloff. This can be seen clearly by examining the ratios of mean uptake of isotope in spleen and lymph nodes on successive days. On day 2 the spleens were roughly

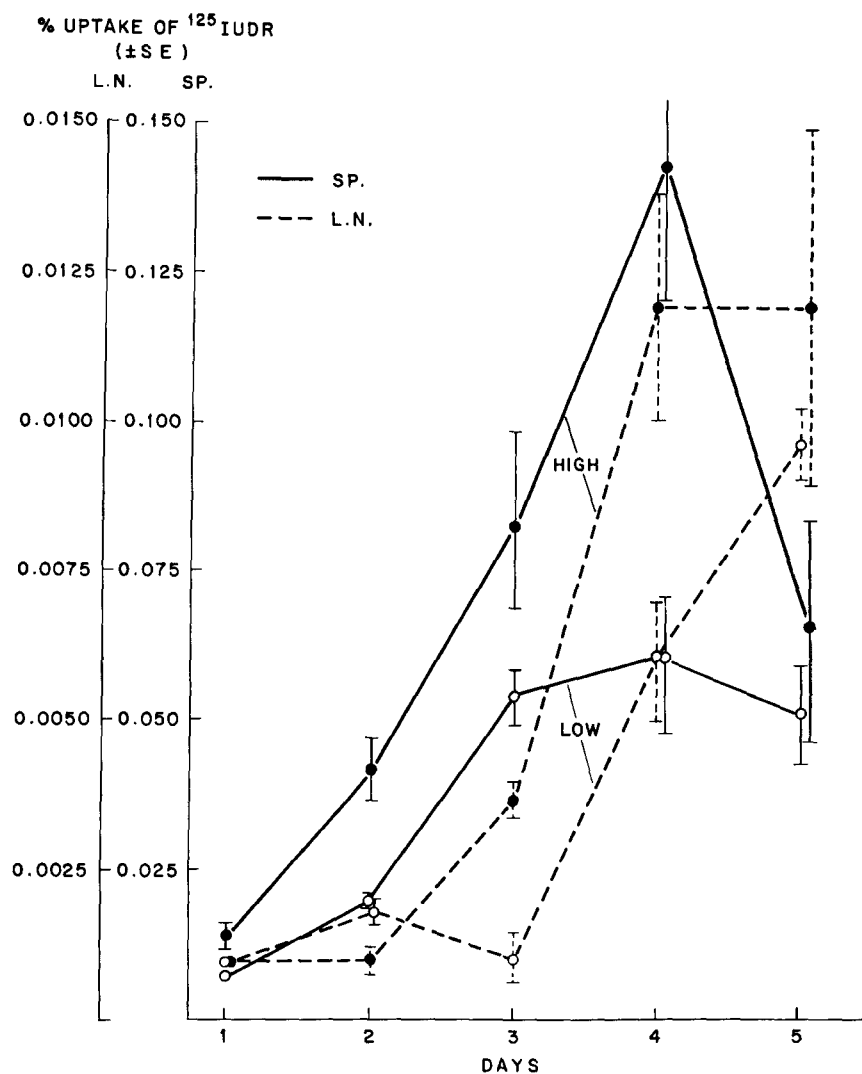


FIG. 2. DNA synthesis of  $4.5$  (*HIGH*) and  $1.5$  (*LOW*)  $\times 10^7$  parental thymocytes in the spleens (*Sp.*) and lymph nodes (*L.N.*) of lethally irradiated  $F_1$  mice on various days after inoculation.

16 times more active; this ratio fell to about nine on days 3 and 4 and fell further to five on day 5.

In Figs. 2-5 we have plotted the results from four individual experiments to illustrate the kinetics of the reactions. There are several points in Fig. 2 which we would like to emphasize. Perhaps most striking is the correlation of a steep

rise in activity with a subsequent steep decline, particularly in the spleen. Note also that the splenic decline did not lead to an increase in lymph node activity.

Comparing the activity of the two cell doses reveals that only on day 4 in the spleen did this reflect, quantitatively, the difference in numbers of cells inoculated. The larger cell dose however, commenced its steep rise in activity 1 day earlier than the lower dose in both the spleens (day 1 vs. day 2) and lymph

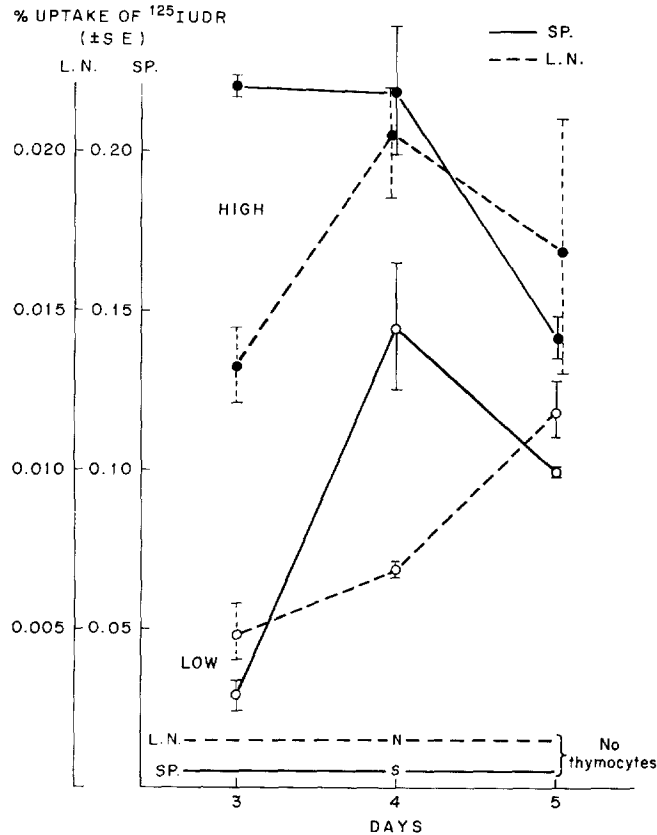


FIG. 3. DNA synthesis of  $2.2$  (*HIGH*) and  $0.7$  (*LOW*)  $\times 10^7$  parental thymocytes in the spleens and lymph nodes of lethally irradiated  $F_1$  mice on various days after inoculation. DNA synthesis in mice not given thymocytes was measured on day 4.

nodes (day 2 vs. day 3). On day 5 the uptake by the high and low doses was almost equal in both spleen and lymph nodes.

In another experiment (Fig. 3) we measured the background DNA synthesis on day 4, and it was extremely low. This experiment illustrates the effect of cell dose on the latent period particularly well. By day 3 the large cell dose had reached its peak in the spleen while the lower cell dose had barely commenced its activity. Similarly, the high cell dose peaked on day 4 in the lymph nodes,

the time at which the low cell dose was only commencing its steep ascent. Also of note is the almost parallel decline of activity of the two cell doses in the spleens between days 4 and 5, in contrast to the lymph nodes, where activity was also declining in one case but in the other it was rising.

In the next experiment (Fig. 4) we tested three different thymocyte doses:  $6 \times 10^7$ ,  $2 \times 10^7$ , and  $7 \times 10^6$  cells. In both the spleen and lymph nodes there was a dose-dependent delay in the start of the sharp ascent of DNA synthesis; the higher the cell dose the earlier the ascent. Similarly, the timing of the plateau

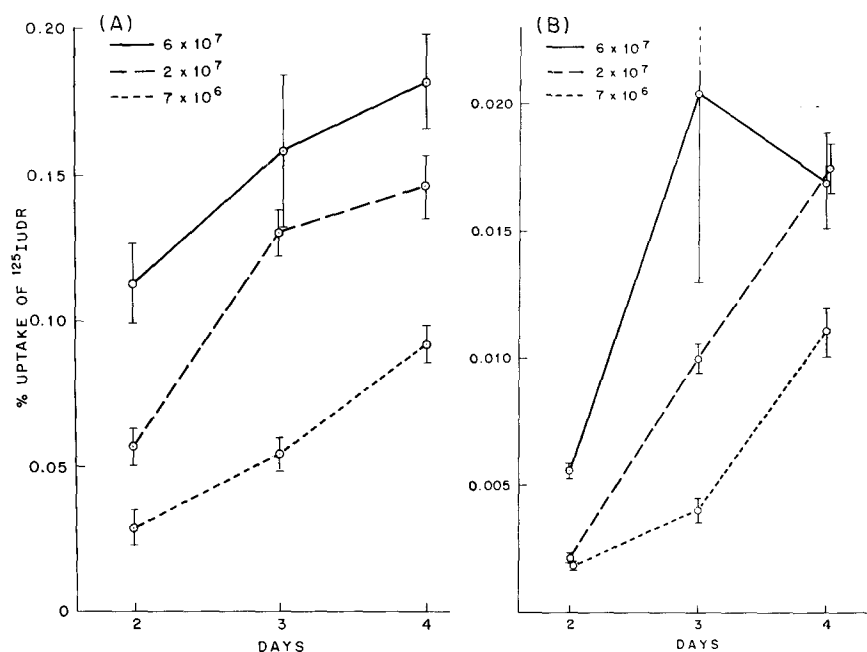


FIG. 4. DNA synthesis of three different doses of parental thymocytes in the spleens (Fig. 4 A) and lymph nodes (Fig. 4 B) of lethally irradiated  $F_1$  mice on various days after inoculation.

or falloff in activity was dose dependent, with an earlier occurrence accompanying a higher dose. The absence of a numerical correlation between dose and DNA synthetic activity was also apparent; i.e., tripling cell doses yielded far less than triple activity. The previously noted independence of activity in spleen and lymph nodes is also well demonstrated in this experiment.

The results of another experiment (Fig. 5) illustrate particularly well the absence of proportional increases in DNA synthesis produced by increasing thymocyte doses. In this experiment a threefold increase in parental thymocyte dose ( $0.8$ – $2.5 \times 10^7$  cells) produced no significant increase in DNA synthesis on any of the 3 days of measurement (a zero-order reaction). Note how, in this

experiment, the steady increase with time of DNA synthesis, in the lymph nodes at the lower thymocyte doses, combined with the shutoff effect at the highest dose, tended to bring the day 5 results of all three groups together. Also of note is the concomitant cessation of DNA synthesis of the high thymocyte doses in the spleen and lymph nodes.

In four further experiments we inoculated recipients with five to six doubling

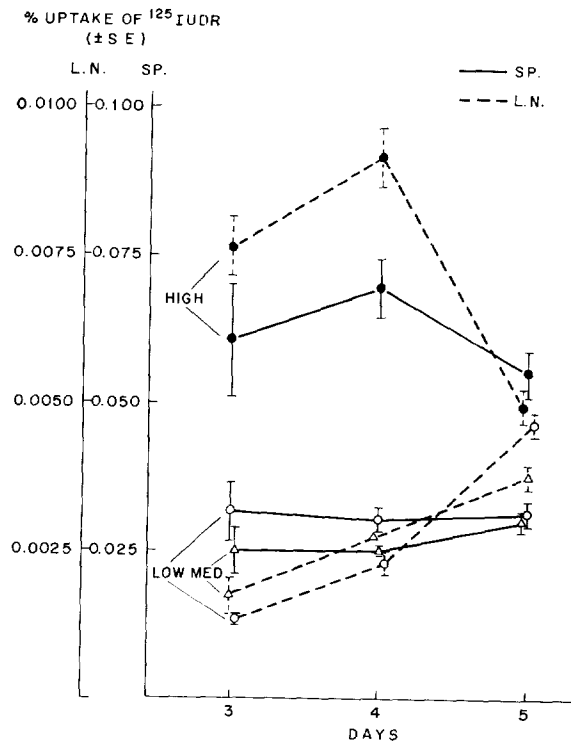


FIG. 5. DNA synthesis of  $7.5$  (*HIGH*),  $2.5$  (*MED*), and  $0.8$  (*LOW*)  $\times 10^7$  parental thymocytes in the spleens and lymph nodes of lethally irradiated  $F_1$  mice on various days after inoculation.

thymocyte doses. The results of one of these, chosen because it is one in which we included control recipients which did not get parental thymocytes, are presented. In Fig. 6, the results are plotted in the standard fashion as uptake vs. time, and in Fig. 7 they are plotted as uptake vs. dose for each of the 4 days of assay. In Fig. 7 the background uptake was subtracted from all results. There are several discernible patterns present which were seen in all four experiments. On day 2 there was no significant increase in DNA synthesis over a wide dose range; an increase occurred only at the higher dose levels, which nevertheless were lower in the spleens than in the lymph nodes. This reflects the previously

noted dose-dependent latent period, and the fact that higher cell inocula are required to shorten it in the lymph nodes than in the spleens. On days 3 and 4 the sharpest increments of DNA synthesis between doses were usually seen, but these were usually less than double. A plateau or in some instances a dip was observed in the day 3 curves between the doses of 3 and  $6 \times 10^7$  cells (spleen) or between 1.5 and  $3 \times 10^7$  (lymph nodes). This plateauing effect was observed in all four experiments on day 3, as in this experiment, or day 4, although the doses between which it occurred varied. The day 5 curve was very similar to that for day 2, except in this experiment the lower doses had completely ceased synthesizing by this time.

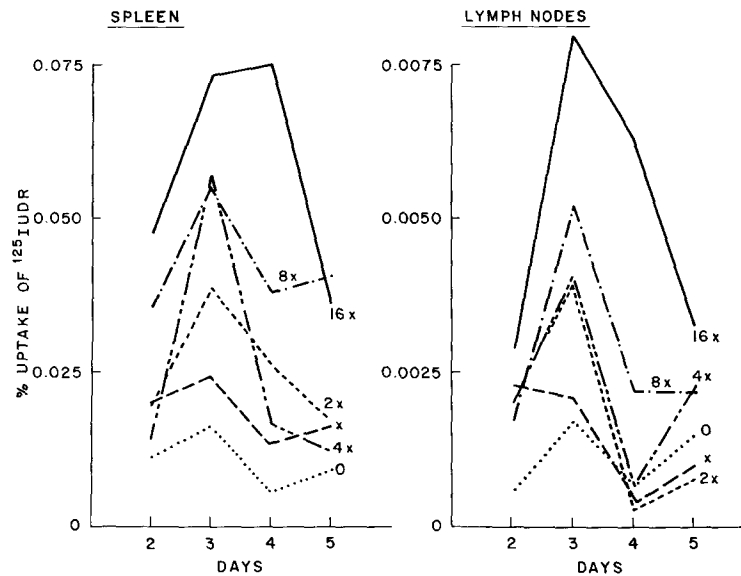


FIG. 6. DNA synthesis of five doubling doses ( $x = 0.75 \times 10^7$ ) of parental thymocytes in the spleens and lymph nodes of lethally irradiated  $F_1$  mice on various days after inoculation.

In all the experiments presented above, we found that increasing the thymocyte dose increased DNA synthesis by only a fraction of the dose increment. In Figs. 8 and 9 the data from these and other experiments in which several cell doses were studied are plotted as the following ratio: (uptake at dose  $Kx$ )/(uptake at dose  $x$ ); Fig. 8 is for twofold ( $K$ ) and Fig. 9 is for threefold dose increases. The relationships were almost always below first order and indeed often approximated zero order or less. Occasionally however, a reaction approaching a second order of response was seen.

In several experiments, we studied doses of  $2 \times 10^7$  and  $3 \times 10^7$  thymocytes. The 50% increase in cell dose more than doubled the DNA synthetic response in both spleen and lymph nodes at five of the six assay points in the experiment reported in Fig. 10 and similar multiple order relationships were seen in others.



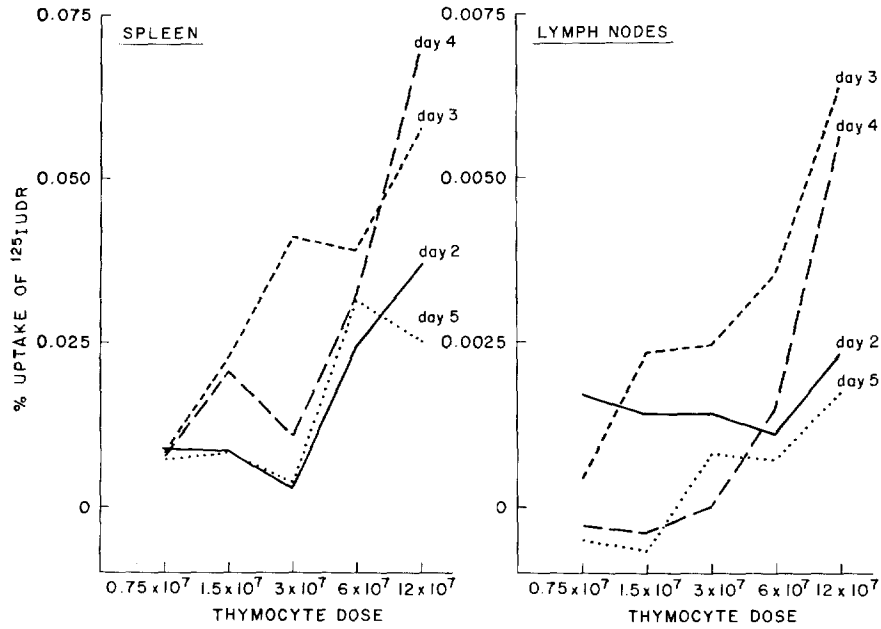


FIG. 7. DNA synthesis in the spleens and lymph nodes of lethally irradiated  $F_1$  mice on different days after the inoculation of five doubling doses of parental thymocytes, minus the DNA synthesis of uninoculated controls.

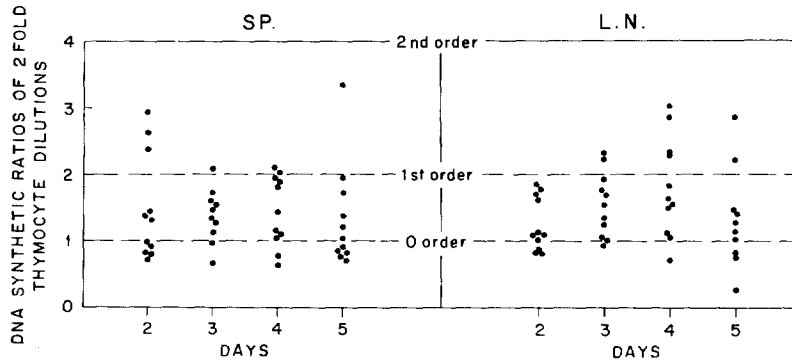


FIG. 8. Ratios of the DNA synthetic response of different doubling parental thymocyte doses to one another in the spleens and lymph nodes of lethally irradiated  $F_1$  mice on various days after inoculation.

In recipients splenectomized before testing, the inoculated cells synthesized more DNA and had a shorter latent period in the nodes than in sham-splenectomized controls (Fig. 11). Since splenectomy has been shown almost to double the numbers of thymocytes localizing in the lymph nodes,<sup>2</sup> these results are

<sup>2</sup> Gershon, R. K., K. Kondo, and E. M. Lance. Regulator T cells. Localization in the spleen. In preparation.

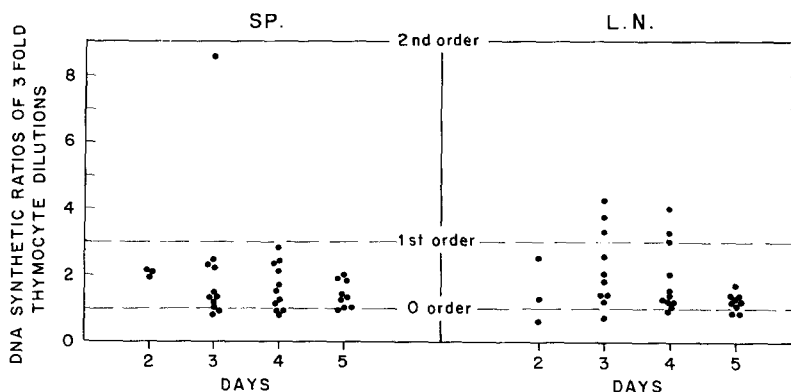


FIG. 9. Ratios of the DNA synthetic response of different tripling parental thymocyte doses to one another in the spleens and lymph nodes of lethally irradiated F<sub>1</sub> mice on various days after inoculation.

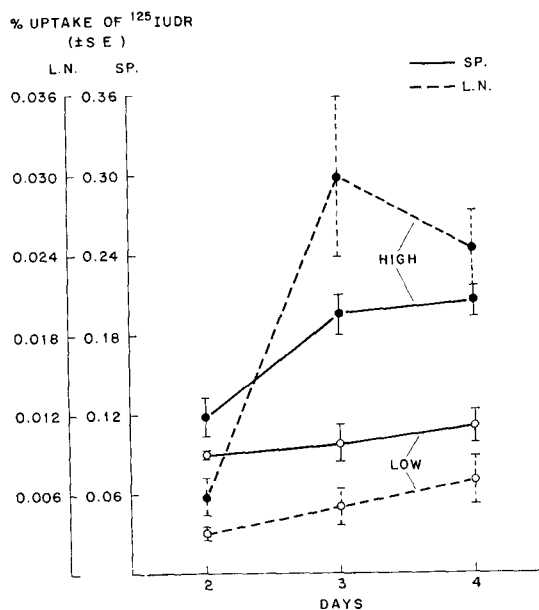


FIG. 10. DNA synthesis of 3 (HIGH) and 2 (LOW)  $\times 10^7$  parental thymocytes in the spleens and lymph nodes of lethally irradiated F<sub>1</sub> mice on various days after inoculation.

consistent with the previously noted shortened latent period produced by increasing cell doses. They also show that the presence of the spleen does not yield an increase of DNA-synthesizing cells in the lymph nodes.

DISCUSSION

We have shown that parental thymocytes synthesize significant amounts of DNA in the lymphoid tissues of lethally irradiated F<sub>1</sub> recipients. Since they do

not do this in similarly prepared syngeneic recipients (17), it is likely that the DNA synthesis reflects their response to the antigens of the reciprocal parent in the  $F_1$  cross. The kinetics of the response are quite interesting. In the spleen, after a latent period of 1-2 days, the thymocytes undergo a burst of DNA synthesis which peaks on day 3 or 4 and then subsides. Their response in the lymph nodes is slightly different; the latent period is longer and the decrease on day 5 less apparent. Although greater in magnitude, the response of parental thymocytes in  $F_1$  spleens is strikingly similar to that of syngeneic thymocytes in

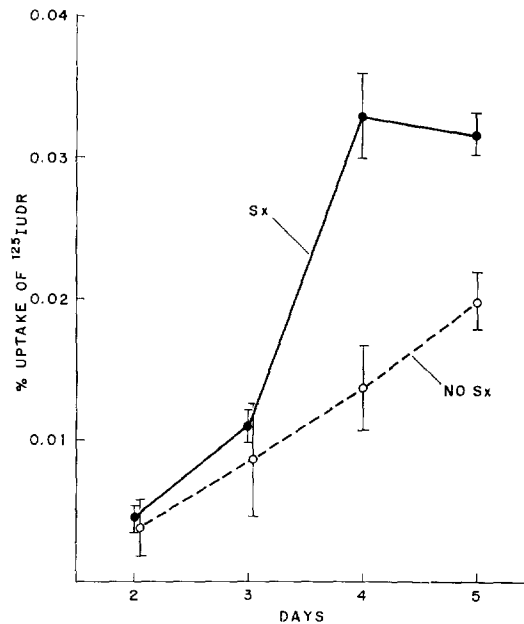


FIG. 11. DNA synthesis of  $1.5 \times 10^7$  parental thymocytes in the lymph nodes of splenectomized (Sx) and sham-splenectomized lethally irradiated  $F_1$  mice on various days after inoculation.

spleens of lethally irradiated mice stimulated with heterologous erythrocytes (17), and to that of chromosomally marked T cells stimulated with antigen in the spleens of syngeneic radiation chimeras (18, 19). In all three cases there is a latent period, a peak response on day 3 or 4, and a subsequent sharp decrease.

The decrease of the response on day 4 or 5 in a GVH situation is particularly intriguing, as several possible explanations offered for this occurrence in other situations can be ruled out here. Shutoff by antibody or other bone marrow-influenced (B) cell products is unlikely to occur in lethally irradiated mice reconstituted only with thymocytes. Elimination of the antigens of the  $F_1$  hosts is equally unlikely. Exhaustion of some reaction product necessary for DNA synthesis cannot explain the decline. Recipient mice can support the synthesis

of much more DNA than the total made by the inoculated thymocytes.<sup>3</sup> Indeed there often was an increase of DNA synthesis in the lymph nodes concomitant with its cessation in the spleen. This particular observation might suggest that the falloff in DNA synthesis was due to migration of the synthesizing cells from the spleens to the lymph nodes. It has been shown that DNA-synthesizing T cells do indeed leave the spleen and join the recirculating pool during GVH reactions (14) and that recirculating cells are a lymph node-seeking population (20). Several observations, however, indicate that such migration cannot account for the sharp fall actually observed. First, in some situations splenic DNA synthesis terminated concomitantly with that in the lymph nodes. Second, prior splenectomy did not diminish DNA synthesis in the lymph nodes, indicating that the major part of the DNA synthesis measured in lymph nodes did not occur in the spleen. Third, no significant migration of chromium-51 labeled parental thymocytes occurs in F<sub>1</sub> hosts during the time-course of these experiments.<sup>2</sup> Fourth, cells taken from the spleens of mice in which the DNA synthetic response is subsiding will undergo a fresh burst of DNA synthesis when transferred to another F<sub>1</sub> host.<sup>4</sup> This last observation also serves to rule out "exhaustive differentiation" (21) as a cause of the cessation of DNA synthesis. Rather, it would seem that potentially reactive cells are indeed present in the spleens of the mice but that their previous response has created a milieu which is inimical to further proliferation. Such an interpretation is supported by the observation of Field and coworkers that, after the initiation of a GVH reaction in F<sub>1</sub> rats, the hosts became refractory to the production of another GVH reaction with cells from either of the parental strains (22). These workers suggested that a humoral factor, most likely enhancing antibody, mediated the refractory state (23). Since few antibody-making cells were present in the mice we studied, we would suggest that an immunosuppressive T cell-released factor, such as that which occurs in antigenic competition (24, 25), is probably the mediator. The temporal association of the cessation of the DNA synthetic response of the thymocytes with the known time of peak depression in antigenic competition experiments (26) supports this possibility.

The interpretation that a suppressive interaction occurs between inoculated thymocytes is strongly supported by our dose-response studies. In almost all cases (we shall comment below on the exceptions) increasing the thymocyte dose by a given multiple increased DNA synthesis by a fraction of that multiple or sometimes not at all. If all the necessary reactants (antigen, nutrients, space, isotope) were present in excess, as indeed they seem to have been, increasing the parental thymocyte dose by a given factor should increase the DNA synthetic response by the same factor (see Materials and Methods). A deviation from this prediction implies some type of cell interaction. Since less than first-

<sup>3</sup> Liebhauer, S. A., and R. K. Gershon. Regulator T cells. In preparation.

<sup>4</sup> Mills, V., and R. K. Gershon. The response of "educated" T cells to H<sub>2</sub>-antigens. In preparation.

order reactions were usually seen, we infer a suppressive interaction. That such suppressed reactions occurred at low as well as high thymocyte doses is good evidence that limiting reaction products were not the cause. Lower than first-order reactions also militate against ascribing the shutoff to "exhaustion," as the cells in these circumstances cannot have expressed their complete mitotic potential.

It is perhaps worth commenting on a possible limitation in interpretation of data obtained by comparing the responses of two different thymocyte doses. Part of the DNA synthetic response measured may have been due to factors other than the thymocyte response to histocompatibility antigen. Thus, an upward shift in baseline DNA synthesis could increase the values of the ratios we measured. However, background DNA synthesis in mice not given any cells after irradiation was very low (Figs. 2 and 6 and reference 17). Indeed, subtraction of background synthesis in the experiment reported in Figs. 6 and 7 did not alter the suppressed nature of the reactions. Another source of error might be DNA synthesis by the added thymocytes in response to something other than histocompatibility antigen. Since thymocytes synthesize very little DNA and also display close to zero-order reactions in the spleens of lethally irradiated syngeneic mice (17), this is probably not a significant factor, particularly since highly suppressed reactions were common when large quantities of DNA were being synthesized. The subtraction of a small amount of background would be most unlikely to influence these results. Indeed, zero-order reactions were often seen and the interpretation of such reactions would not be influenced by the subtraction of any amount of background. Thus we think that the suppressed reactions we have noted are good evidence for negative interactions between thymocytes.

Asofsky, Cantor, and Tigelaar have recently presented evidence for positive interactions between parental T cells in the production of GVH splenomegaly (12). In our studies reactions greater than first order would suggest the occurrence of such reactions. Occasionally, these were found. A possible reason for their infrequent occurrence may be inferred from the experiments in which particularly dramatic effects were noted (Fig. 10): increasing the thymocyte dose from  $2$  to  $3 \times 10^7$  cells (a multiple of 1.5) increased the DNA synthetic response as much as sixfold. It is possible that in order to see positive interactions small dose increments must be studied. If one postulates both positive and negative T cell interactions it would not be surprising to find balancing interactions when comparing larger dose increments. Indeed, Asofsky et al. routinely use very low dose increments to demonstrate their synergistic reactions (12).

The observation that increasing the thymocyte dose decreases the latent period which precedes the sharp increase in DNA synthesis is also good evidence that positive interactions occur between the inoculated cells. This may help explain Wilson and Nowell's observation that preimmunization does not alter

the peak response of mixed lymphocyte reactions but rather causes it to occur earlier (27). These results, supported by others (13, 17), all suggest that positive T cell interactions occur but that their magnitude is regulated by subsequent or concomitant negative ones. It is quite possible that the positive interactions are not entirely specific (as the negative ones appear not to be [24, 26]), which could account for such phenomena as the "allogeneic effect" (28) and "antigenic promotion" (29).

The difference in kinetics of the response in spleen and lymph nodes is interesting. In the latter, the response was significantly delayed and had a decreased tendency to fall sharply on day 5. This second observation may be more apparent than real as the delayed start may have caused a delayed shut-off. Since regeneration of host cells starts to occur on day 6 (17) we were unable to extend our observations past day 5. The delayed response seems, at least in part, to be explained by the decreased numbers of inoculated cells which get to the nodes. When we inoculated the cells into splenectomized recipients we almost doubled the numbers homing to the nodes, and shortened the latent period significantly. This maneuver also produced a shutoff effect on day 5 which was not seen in the mice with intact spleens.

Another difference between the spleen and lymph node response was the magnitude of the response per localizing thymocyte. Studies with chromium-51 indicate that around 50–100 times more thymocytes go to the spleen than to the femoral lymph nodes (20). We found however, that the spleen-localizing cells incorporated less than 10 times the amount of label. A number of factors, none of which is mutually exclusive, could be invoked to explain this difference. Differences in microenvironment and in subpopulations among the inoculated thymocytes seem likely ones and are presently under investigation.

We would like to emphasize that the apparent thymocyte interactions we have noted need not all be direct. For example, macrophages have been shown to release a factor which potentiates the response of thymocytes to certain mitogens (30, 31). Since the release of this factor is partially thymus dependent, it is a good candidate for a causative agent in some of our experiments.

It would appear that our attempt to reduce the complexity of GVH reactions by producing them in situations where host cells do not participate has served to reemphasize their complex nature. Nonetheless, some new insights were gleaned and the technique we have described for the direct measurement of responding cells should be helpful in analyzing them further.

#### SUMMARY

The DNA synthetic response of a wide variety of parental thymocyte doses was studied in the lymphoid tissues of lethally irradiated F<sub>1</sub> mice. The response curves of the thymocytes were strikingly similar in shape to those of T cells responding to antigens, such as sheep red cells, which are more labile than the histocompatibility antigens of the F<sub>1</sub> host. The response was characterized by a

dose-dependent latent period of 1–3 days, followed by a sharp increase in activity and a significant subsequent shutoff. Larger thymocyte doses tended to shorten the latent period. A comparison of the responses of different cell doses to one another indicated that the response usually simulated a suppressed first-order reaction. However, occasional multiple order reactions were observed. We have interpreted these data to indicate that both positive and negative interactions occur between thymocytes, without the mediation of B cell products such as conventional antibodies.

We are grateful to Susan Ryu for her expert technical assistance.

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