REGULATION OF THE IMMUNE RESPONSE

III. Kinetic Differences Between Thymus- and Bone Marrow-Derived Lymphocytes in the Proliferative Response to Heterologous Erythrocytes*

By JOHN W. KAPPLER‡ and MICHAEL HOFFMANN

(From the Department of Biology, University of California, San Diego, La Jolla, California 92037, and Sloan-Kettering Institute for Cancer Research, New York 10021)

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The primary immunization of mice with sheep erythrocytes (SRBC)[1] leads to the stimulation in the spleen of both bone marrow-derived lymphocytes (B cells) and thymus-derived lymphocytes (T cells).

The cellular events underlying the stimulation of B cells have been extensively studied both in vivo (for review see 1) and in vitro (2–6). SRBC-specific precursor cells are selectively stimulated by the antigen with the help of T cells which are also specific for the antigen. These precursors rapidly divide and differentiate into cells secreting specific antibody which generally appear in the spleen with exponential kinetics. The role of cellular proliferation in this process has been established with a number of antimetabolites which inhibit either mitosis (7) or DNA synthesis (8-10) and with lethally high levels of tritiated thymidine (3).

The stimulation of T cells is not as well understood. There is a rapid increase in specific helper T cell activity in the spleens of SRBC-immunized mice (11–13). This "priming" of T cell activity differs from the response of B cells to antigen in that it can be accomplished with very low antigen dose which fails to elicit antibody production (12, 14), is not sensitive to suppression by passively administered anti-SRBC serum (12, 15), and reaches maximal levels within 3 days of immunization (13). In addition to cellular proliferation, roles in the process of priming have been attributed to both the antigen-directed "homing" of specific T cells to the spleen (16) and the activation of inactive T cells by the antigen (17).

We have studied the kinetics of helper T cell priming by SRBC in relation to the response of B cells using a quatitative in vitro technique for the assay of helper activity (13, 18). Experiments utilizing the inhibitor of mitosis, vinblastine, indicate that, although the priming of helper activity precedes the ap-

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[1] *Abbreviations used in this paper:* BSS, balanced salt solution; HRBC, horse erythrocytes; PFC, plaque-forming cells; SRBC, sheep erythrocytes; TNP, trinitrophenyl(ated)-, TRBC, toad erythrocytes.

pearance of antibody-producing cells by as much as 7 day, T cells lag at least 1 day behind B cells in the onset or cellular proliferation. The evidence suggests that the T cells whose activity increases during priming and those which help in the initiation of the primary B cell response may be two different populations.

Materials and Methods

Mice.—8–12-wk old hybrid mice (BDF_1) raised in our own laboratory from C57BL/6 female × DBA/2 male were used in all experiments.

Antigens.—Sheep erythrocytes (SRBC) and horse erythrocytes (HRBC) were obtained from the Colorado Serum Co., Denver, Colo. Trinitrophenylated (TNP) erythrocytes were prepared by the method of Rittenberg and Pratt (19) as modified by Kettman and Dutton (20).

Immunizations.—In vivo immunizations were by tail vein injection in 0.2 ml of balanced salt solution (BSS). Three doses of antigen were used (14): (*a*) High: 2×10^8 SRBC. This dose gives an optimal antibody response but relatively poor T cell priming. (*b*) Intermediate: 2×10^7 SRBC. This dose gives both a good antibody response and T cell priming. (*c*) Low: 2×10^6 SRBC. This dose gives optimal T cell priming but a poor antibody response. In vitro immunizations were 2×10^6 erythrocytes in 0.05 ml of BSS.

Cultures.—Spleen cell suspensions were cultured by the methods of Dutton and Mishell (2) as modified by Kettman and Dutton (20) for TNP-erythrocyte antigens. The cell concentration in all experiments was 10^7 spleen cells per culture.

Drugs.—Vinblastine sulfate was purchased from Eli Lilly and Co., Indianapolis, Ind. Stocks of 1 mg/ml BSS were prepared. The drug was administered intraperitoneally in 0.3 ml of BSS.

Assay of Antibody-Producing Cells.—Cells producing antibody specific for erythrocyte determinants were enumerated by the Jerne hemolytic plaque assay (21), as modified by Dutton and Mishell (2).

TNP-specific antibody-producing cells were assayed by the modification of Rittenberg and Pratt (19) as further modified by Kettman and Dutton (20) using TNP coupled to HRBC in the plaque assay. In each assay a parallel determination was made with TNP-free HRBC. This HRBC "background" was subtracted from the value obtained with TNP-HRBC to calculate the number of TNP-specific plaque-forming cells (PFC). This correction seldom amounted to more than 5%.

Assay for Thymus-Derived Helper Cell Activity.—The helper activity of T cells was assayed by a modification of the techniques of Kettman and Dutton (5, 13, 20). Details of the method are published in the preceding paper in this series (18). Briefly, helper activity was equated with the ability of spleen cells from mice primed with SRBC to enhance the in vitro anti-TNP response of normal spleen cells when immunized with TNP-SRBC. Tritrations were performed in which a varying number of primed cells were added to normal spleen cells keeping the total cell number per culture constant at 10^7. The enhancement of the anti-TNP response above that of normal cells alone in units of anti-TNP PFC per 10^6 recovered culture cells on day 4 of culture was plotted vs. the number of primed cells per culture. The best straight line was fit to the data and the slope of this titration line taken as a measure of the helper activity in the primed cells in units of anti-TNP PFC per 10^6 recovered cells per 10^6 primed cells.

RESULTS

The Kinetics of the Priming of Helper T Cell Activity and the Anti-SRBC PFC Response.—The spleens of mice were examined at various times after immuniza-

tion with SRBC for anti-SRBC PFC and for SRBC-specific helper activity. The results are shown in Fig. 1. At either a high (2×10^8) or intermediate (2×10^7) dose of SRBC, PFC begin to appear in the spleen at about 1 day after immunization and increase exponentially until a maximum is reached after 4 days. The increase in helper activity after an intermediate (2×10^7) or low (2×10^6) dose of SRBC is evident by day 1, substantial by day 2, and nearly maximal by day 3.

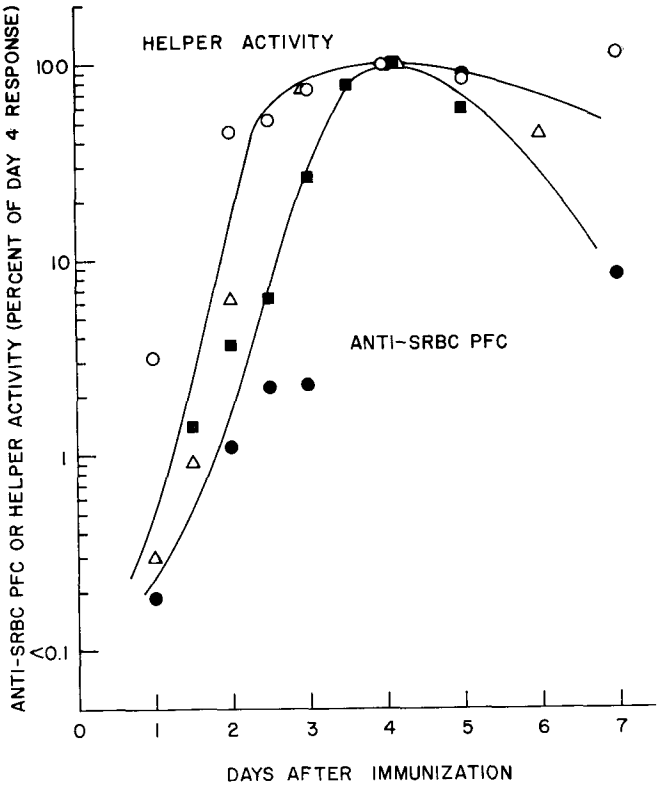

FIG. 1. Kinetics of the priming of SRBC-specific helper T cells and the development of anti-SRBC PFC. Results of three experiments are shown. Groups of three mice were immunized with 2×10^6 (triangles), 2×10^7 (circles), or 2×10^8 (squares) SRBC at various times before sacrifice. At sacrifice spleens were assayed for anti-SRBC PFC (closed symbols) and/or for the presence of SRBC-specific helper activity (open symbols) as described in Materials and Methods. Anti-SRBC PFC are the geometric means of determinations made on individual spleens within each group. Helper activity was determined using several concentrations of a pooled suspension of the spleens of each group. For ease of comparison the results are normalized to 100% at day 4. The actual anti-SRBC PFC response on day 4 in PFC/10^6 spleen cells were: 2×10^8 SRBC, 2,150; 2×10^7 SRBC, 569. The actual helper activities on day 4 in anti-TNP PFC/10^6 recovered culture cells/10^6 primed spleen cells were: 2×10^6 SRBC, 1,381; 2×10^7 SRBC, 405.

The Effect of Vinblastine on the Priming of Helper T Cell Activity and the Anti-SRBC PFC Response.—In order to study the role of cellular proliferation in the priming of helper activity the mitotic inhibitor, vinblastine, was used. Vinblastine was given to mice at various times after immunization with SRBC. The spleens of these animals were then examined for anti-SRBC PFC and SRBC-specific helper activity on day 4 after immunization, a time when both are maximal in untreated controls. The results are shown in Fig. 2. When given with the

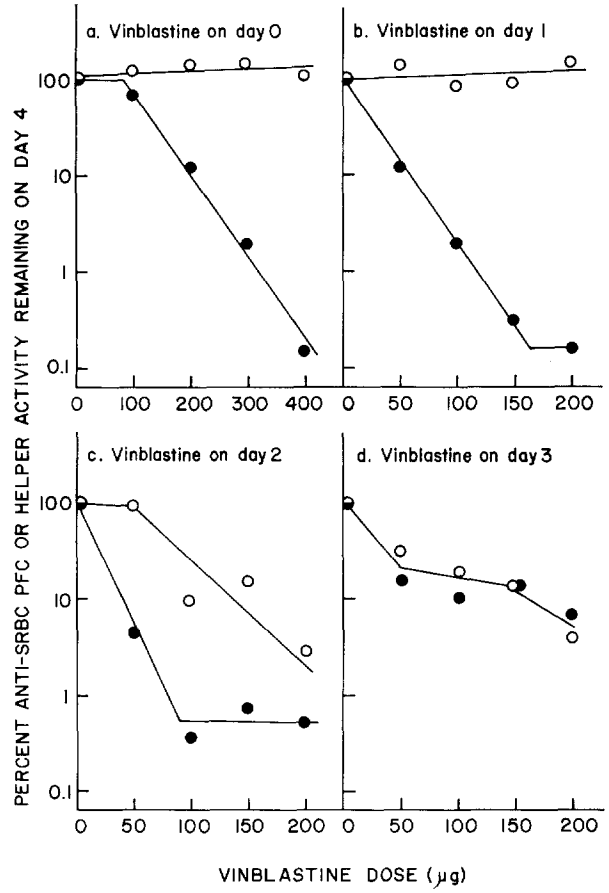

FIG. 2. Effect of vinblastine on the priming of SRBC-specific helper T cells and the development of anti-SRBC PFC. The results of four experiments are shown. In each experiment groups of four animals were given 2×10^7 SRBC (day 0) and a varying dose of vinblastine on either day 0 (exp. *a*), day 1 (exp. *b*), day 2 (exp. *c*), or day 3 (exp. *d*). On day 4 the animals were sacrificed and their spleens assayed for anti-SRBC PFC (●) and SRBC-specific helper activity (○) as in Fig. 1. Results are shown as the percent of the responses in the control animals receiving no vinblastine. The mean anti-SRBC PFC responses in the control groups in PFC/10^6 spleen cells were: exp. 2, 545; exp. *b*, 348; exp. *c*, 170; and exp. *d*, 194. The helper activity in the control groups in anti-TNP PFC/10^6 recovered culture cells/10^6 primed spleen cells were: exp. *a*, 525; exp. *b*, 918; exp. *c*, 472; and exp. *d*, 1,210.

antigen (day 0) doses of greater than 100 μg of vinblastine suppress the appearance of anti-SRBC PFC but have no effect on the priming of helper activity (Fig. 2 *a*). Similar results are obtained when the vinblastine is given 1 day after the antigen except that the anti-SRBC PFC response is inhibited by much smaller doses of vinblastine (Fig. 2 *b*). When given 2 days after the antigen, vinblastine inhibits the anti-SRBC PFC response at relatively small doses, whereas doses above 50 μg inhibit the priming of helper activity (Fig. 2 *c*). Finally when given 3 days after the antigen both the anti-SRBC PFC response and the priming of helper activity are suppressed equally well by vinblastine (Fig. 2 *d*).

The data in Fig. 2 suggest that helper T cells lag behind B cells in the onset of cellular proliferation after immunization. This point is better seen in Fig. 3 in which some data from Fig. 2 and data from a number of other experiments are plotted as the percent of the anti-SRBC PFC response or helper activity remaining on day 4 vs. the time after immunization at which a dose of 150 μg of vinblastine is given. At an immunizing dose of either 2×10^7 or 2×10^8 the anti-SRBC PFC response is inhibitable by vinblastine immediately after immunization. On the other hand the priming of helper T cell activity cannot be inhibited by vinblastine until about $1\frac{1}{2}$ days after immunization.

Specificity of the Effect of Vinblastine.—In order to interpret the results shown above it was necessary to show that the effect of vinblastine was specific for the immunizing antigen. The specificities of the effect on T cells and B cells are shown in Table I and Table II, respectively.

In Table I two groups of four mice (A and B) were given SRBC on day 0 and a non-crossing-reacting antigen, TRBC, on day 2. On day 2.5, 150 μg of vinblastine was given to group B to suppress the priming of SRBC-specific helper activity. On day 5 the spleens of both groups were assayed for both SRBC- and TRBC-specific helper activity. The results show that as expected only the priming of SRBC-specific helper activity is inhibited by the vinblastine.

In Table II groups of eight mice were given SRBC on day 0 followed by either 0, 50, or 100 μg of vinblastine on day 1. On day 2 all mice were given a second non-cross-reacting antigen, TNP-TRBC. On day 4 the spleens of half of each group were assayed for anti-SRBC PFC and on day 6 the spleens of the second half assayed for anti-TNP PFC. (The anti-TNP response was assayed rather than the anti-TRBC response, since TRBC are poor indicators in the plaque assay.) The results show that only the PFC response to SRBC is suppressed by the vinblastine.

DISCUSSION

We have studied the kinetics of the response to the antigen SRBC in mouse spleen of both helper T cells and the B cell precursors of PFC. After a primary immunization with SRBC anti-SRBC PFC began to appear in the spleen after 1 day and increased exponentially until about day 4. The priming of helper T

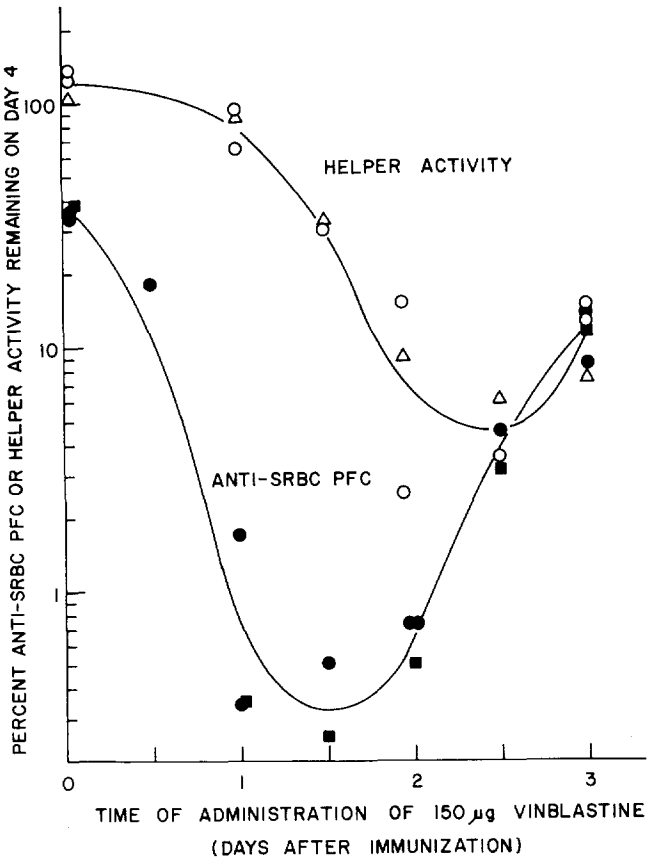

FIG. 3. Kinetics of the proliferative response of T cells and B cells to SRBC. The results of a number of experiments are shown. In each experiment groups of three to four mice were given either 2×10^6 (triangles), 2×10^7 (circles), or 2×10^8 (squares) SRBC on day 0. At various times afterwards 150 μg of vinblastine was administered. On day 4 the animals were sacrificed and their spleens assayed for anti-SRBC PFC (closed symbols) and/or SRBC-specific helper activity (open symbols) as in Fig. 1. Results are reported as the percent of the responses in groups of control mice which received no vinblastine. The mean anti-SRBC PFC responses in the control groups varied from 76 to 1,210 PFC/10^6 spleen cells in the various experiments. The SRBC-specific helper activity in the control groups varied from 348 to 1,992 anti-TNP PFC/10^6 recovered culture cells/10^6 primed spleen cells in the various experiments.

cell activity was more rapid with maximal activity reached by about day 3. These results are generally in agreement with those of others (2, 12, 13, 22). We also compared the role of cellular proliferation in the responses of these two cell types and found that they show quite different kinetics. Using sensitivity to the mitotic inhibitor, vinblastine, as an indicator of proliferation, we determined

TABLE I

Specificity of the Effect of Vinblastine on T Cells

Group*	Protocol			Assay for helper activity‡ Day 5	
	First antigen Day 0	Second antigen Day 2	Vinblastine Day 2.5	SRBC-specific (PFC/10^6 per 10^6)	TRBC-specific (PFC/10^6 per 10^6)
A	2×10^6 SRBC	8×10^5 TRBC	—	304 100%§	244 100%
B	2×10^6 SRBC	8×10^5 TRBC	150 μg	50 16%	268 110%

* Four mice per group.

‡ Spleens pooled within each group before assay.

§ Percent of group A.

TABLE II

Specificity of the Effect of Vinblastine on B Cells

Group	Protocol				
	First antigen Day 0	Vinblastine Day 1	Second antigen Day 2	Anti-SRBC response PFC/10^6 Day 4	Anti-TNP response PFC/10^6 Day 6
A	2×10^7 SRBC	—	2×10^7	377* (212–677)‡	146 (118–177)
			TNP–TRBC	100%§	100%
B	2×10^7 SRBC	50 μg	2×10^7	111 (77–161)	166 (131–193)
			TNP–TRBC	29.5%	114%
C	2×10^7 SRBC	100 μg	2×10^7	12.4% (7.4–23.9)	203% (157–248)
			TNP–TRBC	3.3%	139%

* Geometric mean of determinations in four animals.

‡ Range.

§ Percent of group A.

that B cells begin to divide under antigen stimulation at least one day before helper T cells. The peak of sensitivity to vinblastine of the B cell response is between day 1 and day 2 when PFC are assayed on day 4. The peak of sensitivity of the T cell response under the same conditions does not occur until between day 2 and day 3. We obtained similar results with a number of immunizing doses.

We had to consider several trivial explanations for our results. It might have

been that the sensitivity of T cells and B cells to vinblastine was due to some nonspecific effect on the drug on all cells in the spleen, both responding and nonresponding, or perhaps, in the case of T cells, the effect of vinblastine may have been indirectly due to the carry-over of the drug into the culture assay for helper activity. The necessity for the proper scheduling of the administration of antigen and vinblastine for the maximal effect (see Fig. 3) made these possibilities seem unlikely; however, they were eliminated by the control experiments described in Tables I and II, showing the specificity of the effect of vinblastine on SRBC-stimulated T cells and B cells.

Another possibility was that the effect of vinblastine on T cells and B cells may not reflect a difference in the kinetics of proliferation, if the proliferating T cells are preferentially resistant to the action of vinblastine for at least 1 day. We are unaware of any evidence to support this possibility and consider it highly unlikely, although it cannot be eliminated in a strict sense on the basis of the data presented here.

Our results are in marked contrast to those of Segal et al. (23), who reported that the priming of helper activity to rabbit serum albumin in mice is sensitive to vinblastine only during the first 24 h, a time when we have seen no sensitivity even to high doses of vinblastine in the priming of helper activity to SRBC. These differences in our results are not at present resolved.

Our results are, however, in general agreement with the work of Byfield and Sercarz (9), who describe the existence of what they call "quick response" memory in the spleens of mice primed with SRBC. This memory, seen between 1 and 10 days after priming, is most likely due to helper T cell stimulation, since it occurs at low priming doses which have been shown by others to be preferential for T cells (12, 14, 24). The establishment of this memory is sensitive to the inhibitors of DNA synthesis, methotrexate, and hydroxyurea, particularly between days 1 and 4 after priming.

There are other reports of the rapid proliferation of T cells in response to stimulation with SRBC. Davies et al. (25, 26) performed experiments with adult thymectomized mice which were irradiated, reconstituted with bone marrow, and grafted with a thymus lobe from mice bearing the T6T6 chromosomal marker. They demonstrated that a sizable proportion of the mitotic cells found in the spleens of these animals after immunization with SRBC were of the thymus graft origin, with the peak of the proliferative response coming 3 days after the antigen was administered.

A number of workers have shown the presence of helper activity in the spleens of irradiated mice which have been reconstituted with thymus cells and SRBC (27–29). The development of this activity, sometimes referred to as "thymus education" has been shown by Ito and Cudkowicz (30) to be sensitive to vinblastine during day 1 and day 2 after reconstitution. Whether or not these results can be compared to those obtained with priming is unclear, since thymus education differs from priming in a number of properties, such as the kinetics

of the appearance of helper activity, the dose of antigen required, and perhaps the stage of differentiation of the stimulated cells.

Mishell and Dutton (3) have demonstrated that the precursors of anti-SRBC PFC are maximally sensitive to high doses of tritiated thymidine (specifically lethal to cells synthesizing DNA) between 1 and 2 days after stimulation in vitro with SRBC. On the other hand a large number of workers (31, 32, for reviews see 33, 34) have shown that T cells populations stimulated in vitro with mitogens or antigens show their maximal proliferative response about 3 days later, again indicating a difference in the kinetics of the response of the two cell types.

The demonstration that in the primary response to SRBC, T cells lag at least 1 day behind B cells in the onset of cellular proliferation, leads to the question of the nature of the T cell which has been demonstrated to participate in the initiation of the primary B cell response. Our data would suggest either that the T cell which initiates the primary response does so before proliferating or that there are two populations of T cells involved. This second possibility has been suggested by Raff and Cantor (35) in a recent study indicating the presence of two subpopulations of T cells in mice. Although the evidence is incomplete, a model emerges which can be used to interpret the data. One subpopulation, T1, is relatively immature and is preferentially found in the spleen. These cells do not recirculate and are not removed by thoracic duct drainage. T1 cells are apparently shortlived and of recent thymus origin in that they disappear from the animal within 2 to 6 wk after adult thymectomy. The other population of T cells, T2, is more mature and found preferentially in the circulation and lymph nodes. They recirculate and are removed by thoracic duct drainage. T2 cells are long-lived, being detectable in the animal 30 wk after thymectomy. Raff and Cantor (35) propose that priming is the antigen-driven pfoliferation and differentiation of T1 cells to T2 cells which are the active helper T cells. The primary B cell response is considered to be initiated by a few preexisting T2 cells which arise during the development of the animal through the stimulation of T1 cells by environmental antigens which cross-react with SRBC. Our data is consistent with this model.

Raff and Cantor (35) predict that adult thymectomy should affect mainly those T cell functions requiring T cell priming, but not the primary B cell response. We have preliminary evidence that this is indeed the case.[2] Whereas, the in vivo and in vitro primary responses ro SRBC are normal up to 15 wk after adult thymectomy, the ability to prime T cells for helper activity or for delayed hypersensitivity decreases rapidly within a few weeks. The better understanding of these observations will have to await further evidence on the nature and the relation between these two T cell populations.

The data in Figs. 1 and 3 indicate that although helper T cells are undergoing

[2] Hunter, P., D. Jacobs, J. Kappler, and E. Lord. Manuscript in preparation.

rapid division between day 3 and day 4 after immunization, there is no substantial increase in the concentration of helper activity in the spleen during this time. This observation might be explained by the saturation of the spleen with primed T cells by day 3, followed by the exit of excess cells. Consistent with this interpretation is the work of Sprent et al. (16), who have demonstrated that T cells leave the circulation (and presumably lodge in the spleen and lymph nodes) shortly after immunization with SRBC, but return in increased numbers between day 3 and day 5.

Recent evidence has shown that a number of manipulations can lead preferentially to a cellular rather than a humoral immune response. Low doses of antigen (12, 14, 24), chemically modified antigens (36, 37), antigens which cross-react at the T cell level only (18, 38–40), and antigens given in the presence of passively administered antiserum (12, 15) have all been used to initiate a T cell response in the absence of significant antibody production. Our data indicate yet another method. The proper selection of the dose of vinblastine and the timing of administration leads to a normal T cell response to SRBC in the virtual absence of antibody production (Fig. 3). These techniques may yet prove useful in clinical situations, such as cases involving certain cancers where the production of "blocking antibody" can interfere with the successful T cell-mediated rejection of a tumor (41).

SUMMARY

The kinetics of the in vivo response to SRBC was studied in mouse spleen at both the B cell and T cell levels. The B cell response was assayed by following the appearance of antibody-secreting cells in the spleen using the hemolytic plaque assay. The T cell response was monitored by following the increase in or "priming" of helper activity in the spleen using a quantitative in vitro assay. The role of cellular proliferation in both responses was established with the inhibitor of mitosis, vinblastine.

The results show that, although the development of T cell activity precedes that of anti-SRBC PFC by as much as 1 day, T cells lag at least 1 day behind B cells in the onset of cellular proliferation.

The evidence suggests either that the helper T cell which proliferates in response to SRBC does so after helping in the initiation of the primary B cell response or that the proliferative T cell response and the initiation of the primary B cell response involve two different subpopulations of T cells.

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