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Induction of Latent Immunological Memory in Genetically Nonresponsive Mice (42583)

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Abstract. C57BL/10 mice exhibit major histocompatibility complex linked nonresponsiveness to hen egg white lysozyme (HEL). When these animals are primed with HEL in Freund's complete adjuvant (FCA), their secondary splenic plaque forming cell responses to aqueous HEL challenge are minimal to nonexistent. This notwithstanding, we show here that concomitant priming with both HEL and keyhole limpet hemocyanin (KLH) leads to an enhanced response to the HEL component following secondary challenge with an HEL–KLH conjugate. This enhancing effect can be transferred by nylon wool nonadherent spleen cells from HEL/FCA primed animals. Adoptive transfer studies with fractionated spleen cell populations suggest also that B cells are primed in these animals. Thus, animals which are incapable of mounting a secondary response to this antigen nevertheless appear to be primed at both the T-cell and B-cell levels following exposure to the antigen in FCA. The implications of this finding are discussed.

Much of our current understanding of the mechanisms which regulate immune responsiveness derive from studies of genetically determined immune response capabilities. The responses of several different species to many model antigens have been shown to be under genetic control. For the most part these response capabilities have been shown to be linked to the major histocompatibility complex (MHC) of the species in question, although in a few instances linkage to the immunoglobulin allotype has been demonstrated (1).

At present it is clear that at least two distinct mechanisms are operative in MHC linked nonresponsiveness. In one, exposure of the animal to the antigen in question leads to the induction of suppressor cells specific to that antigen (2). These suppressors then limit any further responsiveness of the animal. The second mechanism, which remains ill understood, involves some defect in recognition of the antigen at the level of the antigen-presenting cell (3, 4). Whether this results from nonexpression of appropriate receptors, lack of "processing," or some negative consequence of antigen interaction with class II molecules is unknown (5, 6).

We have been studying the mechanisms underlying ir gene control of the response of C57BL/10 mice to hen egg white lysozyme (HEL). This is a complex system in which nonresponsiveness is compartmentalized, being predominantly expressed in the central lymphoid organs (7). Both suppressor-cell involvement (7, 8) and defects at the antigen presentation level (9, 10) have been demonstrated. We report here still another aspect of the complexity of this system. Thus, nonresponsiveness notwithstanding, exposure of B10 mice to HEL in Freund's complete adjuvant (FCA) leads to the appearance in their spleens of primed B and T cells with apparent HEL specificity.

Materials and Methods. *Animals.* Balb/c ♀ × A/J ♂ (CAF_1) and C57BL/10 SgSn (B10) mice were purchased from Jackson Laboratories, Bar Harbor, Maine.

Antigens. Hen egg white lysozyme was purchased from Miles Laboratories, Elkhart, Indiana. Keyhole limpet hemocyanin (KLH) was prepared as described by Campbell *et al.* (11). The keyhole limpets (*Megathuria crenulata*) were purchased from Pacific Biomarine, Venice, California. FCA containing *Mycobacterium tuberculosis* strain H37 Ra was obtained from Difco Laboratories, Detroit, Michigan.

HEL was conjugated with lipopolysaccharide (LPS) as described previously (12). HEL was conjugated with KLH via carbodiimide-promoted amide bond formation as follows: to 50 mg of KLH dissolved in 3 ml of 0.5 *M* NaCl was added 50 mg of HEL followed by

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25 mg of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (Aldrich, Milwaukee, WI). The mixture was stirred for 4 hr at room temperature and the proteins were then precipitated by the addition of saturated ammonium sulfate to effect 50% saturation. The resulting precipitate was sedimented by centrifugation and the pellet was redissolved in 1 ml of 0.5 *M* NaCl. This solution was fractionated on a 2.5 × 50.0 cm Sephadex G-75 column at a flow rate of 2.2 ml/min of 0.5 *M* NaCl. Two absorbance peaks (280 nm) were obtained, the first of which contained KLH. The KLH-containing fractions were pooled, precipitated with ammonium sulfate, pelleted, and redissolved in 0.5 *M* NaCl. The final product was assayed for the presence of uncoupled HEL by immunoelectrophoresis in a 1% agarose gel using 0.05 *M* barbital buffer, pH 8.0. Following electrophoresis, immunoprecipitation was effected by the addition of hyperimmune murine anti-HEL antibodies. Only one precipitin band was obtained and this band was located in the area of the gel which contained the KLH as determined by its blue color. No precipitation was seen in that area of the gel to which the control free lysozyme migrated.

Immunizations, cellular manipulations, and assays. Primary immunizations consisted of 100 μg of each antigen, emulsified in FCA and administered intraperitoneally (0.1 ml). Where two antigens were utilized they were administered individually but at the same time. HEL–LPS priming consisted of 20 μg HEL–LPS in 0.1 ml of saline administered intraperitoneally. Secondary challenges consisted of 100 μg of each antigen dissolved in 0.1 ml of saline. Where two antigens were utilized they were admixed prior to administration.

Spleen cell preparation, T- and B-lymphocyte isolation, adoptive cell transfer procedures, and assay procedures were all performed as previously described (12). Briefly, T cells were enriched by passage over nylon wool and B cells were prepared by treatment of whole spleen with rabbit-antibrain associated θ plus complement (13). All cell transfers utilized 5×10^7 cells of the type indicated. Where admixtures of cells were administered, 5×10^7 of each type were utilized. All recipients received 600 R X-irradiation on the day of transfer. Antibody-forming cells were detected by the Cunningham and Szenberg modification of the Jerne plaque assay. No immunoglobulin M (IgM) plaques were detected in these experiments. Consequently, only immunoglobulin G (IgG) plaques are recorded. Background (anti-red blood cell) responses constituted less than 1% of plaques in all cases.

Results. *A lysozyme–KLH conjugate effects helper-cell–B-cell interaction.* Presented in Table I are the results of an experiment designed to determine whether the HEL–KLH conjugate functions to bridge KLH specific helper cells to HEL specific B cells. As in earlier studies we took advantage of the relative radioresistance of primed helper-T-cell function (13, 14). Groups of CAF_1 mice were primed with either KLH or saline in FCA. One month later, these animals were irradiated with 600 R and grafted with anti-T-cell-treated whole spleen derived from CAF_1 donors which had been primed 1 month earlier with HEL–LPS. The recipients were then challenged with either a mixture of HEL and KLH or the HEL–KLH conjugate and their anti-HEL splenic plaque-forming cell (PFC) responses were assessed 6 days later. As can be seen, only those recipients which had been primed with KLH and which were challenged with the HEL–KLH conjugate were able to respond. The HEL/KLH mixture was ineffective in eliciting a response. Thus, the HEL–KLH conjugate was effective in "bridging" between the primed, radioresistant KLH-specific helper cells and the adoptively transferred, HEL-specific B cells.

HEL-KLH induces an anti-HEL response in KLH-primed nonresponder mice: Concurrent HEL priming enhances this response. Having demonstrated that HEL–KLH could effectively function as a "hapten-carrier" con-

TABLE I. CAF_1 MICE: HEL-SPECIFIC PFC GENERATED IN KLH-PRIMED, 600 R X-IRRADIATED RECIPIENTS OF HEL-PRIMED B CELLS

Priming antigen	Challenge antigen	PFC/10^6 spleen cells[a]
Saline/FCA	HEL–KLH	85 ± 10
KLH/FCA	HEL–KLH	4508 ± 177
Saline/FCA	HEL + KLH	2 ± 1
KLH/FCA	HEL + KLH	3 ± 1

[a] Means ± SE.

jugate, we next sought to determine whether this conjugate was capable of circumventing the nonresponsiveness of B10 mice to HEL. Accordingly, groups of B10 mice were primed with FCA containing saline, HEL, KLH, or a mixture of HEL and KLH. One month later these animals were challenged with HEL–KLH and their splenic anti-HEL PFC responses were assessed 6 days thereafter. As can be seen in Fig. 1, priming with either saline or HEL was ineffectual. On the other hand, in those animals primed with KLH a respectable anti-HEL response was obtained. Surprisingly, concomitant priming with HEL in addition to KLH led to an enhancement of this response. Since this latter group was included to probe for putative suppression of the response to the conjugate (see Discussion) this effect was quite unexpected. We accordingly set up a series of experiments to further explore the phenomenon.

Cellular basis of HEL "priming" effect in B10 mice. In order to probe the cellular basis of this priming effect it was necessary to set up a series of adoptive cell transfer experiments utilizing fractionated cell populations from the HEL-primed animals. Furthermore, in order to assess for putative T-cell effects it was necessary to have a known source of HEL-primed B lymphocytes. Since previous studies had indicated that HEL complexed with LPS induces a strong anti-HEL primary response in B10 mice (15) we reasoned that such animals might serve as a source of primed B cells. To test for this, B10 mice were primed with saline or

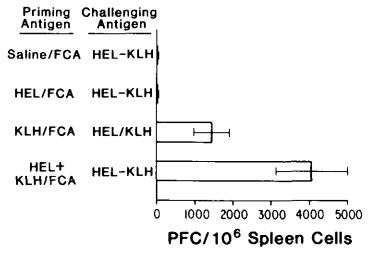

FIG. 1. Effects of preimmunization on the responses to HEL–KLH of C57BL/10 mice. C57BL/10 mice were primed (100 μg protein) as indicated, rested for 1 month, and then challenged with the HEL–KLH conjugate (100 μg). HEL-specific PFC responses were measured 6 days after challenge. Limit bars represent standard error of the mean.

TABLE II. C57BL/10 MICE: HEL-SPECIFIC PFC GENERATED TO HEL–KLH CHALLENGE IN PRIMED, 600 R X-IRRADIATED RECIPIENTS OF HEL–LPS-PRIMED B CELLS

Recipients primed with	B cell donor primed with	PFC/10^6 spleen cells[a]
Saline FCA	HEL–LPS	13 ± 2
KLH/FCA	HEL–LPS	343 ± 18
KLH/FCA	Saline	40 ± 16

[a] Means ± SE.

KLH in FCA, rested for 1 month, irradiated with 600 R, and grafted with HEL primed B cells. The latter consisted of anti-T-cell-treated spleen cells from B10 mice which had been injected 1 month previously with either saline or the HEL–LPS complex. The recipients were then challenged with the HEL–KLH conjugate and their anti-HEL responses were assessed 6 days later. As can be seen in Table II, those animals which had been primed with KLH and grafted with HEL–LPS B cells responded to the HEL–KLH, whereas the control groups did not. Thus, HEL–LPS-primed B10 mice could be utilized as a source of HEL-primed B cells.

We next set up an experiment to assess the effect of HEL/FCA priming on B10 T cells. This involved a protocol essentially identical to that of the previous experiment with the added manipulation of mixing in whole or fractionated spleen from HEL/FCA-primed donors. Thus, a large group of B10 mice were primed with KLH in FCA, irradiated with 600 R, and then grafted with anti-T-cell-treated spleen cells from HEL–LPS-primed donors as the source of primed B cells. Different groups of these recipients were additionally grafted with whole spleen or the B- or T-cell fraction from the HEL/FCA-primed donors. All of these recipients were then challenged with HEL–KLH. The results of this experiment are presented in Fig. 2. When compared to the baseline response obtained with the naive spleen supplement, the B-cell fraction (anti-T-cell treated) from the HEL/FCA-primed donors was if anything suppressive, whereas the whole spleen population enhanced the response. Furthermore, the nylon wool nonadherent population from the HEL/FCA-primed animals had an even greater enhancing

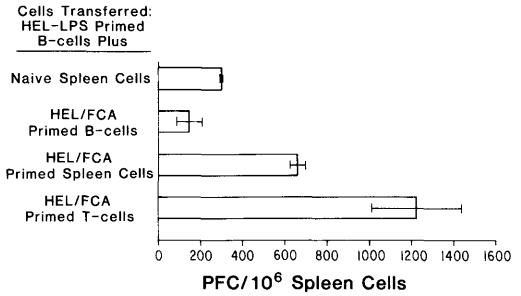

FIG. 2. Response to HEL–KLH of KLH-primed recipients of HEL–LPS-primed B cells plus various HEL/FCA-primed cellular populations. C57BL/10 donors were primed with the indicated antigen and rested 1 month prior to harvesting of their spleen cells. T- and B-cell fractions were prepared as indicated in the text. HEL–LPS-primed B cells were added to each of the other donor populations prior to transfer. These mixtures were then grafted into 600 R X-irradiated recipients which had been primed with 100 μg KLH in FCA 1 month earlier. All of the recipients were then challenged with 100 μg HEL–KLH and their HEL-specific PFC responses were assessed 6 days later. Limit bars represent standard error of the mean.

effect, indicating that this was in fact a T-cell effect.

Having demonstrated enhancement by T cells from HEL/FCA-primed B10 mice, it became relevant to question the status of B cells in such animals. Accordingly, a series of KLH-primed recipients were irradiated with 600 R and then grafted with HEL/FCA-primed whole spleen, the B-cell fraction, the T-cell fraction, or these two fractions recombined (none of these animals received HEL–LPS-primed B cells). These recipients, along with recipients of naive spleen cells (saline/FCA primed), were then challenged with HEL–KLH and their anti-HEL responses were assessed. As can be seen in Fig. 3, both whole spleen and the reconstituted splenic lymphocyte fractions conferred anti-HEL responsiveness, whereas restoration by either fraction alone was minimal. This rather strongly suggested that primed B cells were present in the HEL-primed spleen.

Spleen cells from individual HEL/FCA-primed donors enhance the anti-HEL–KLH response of individual KLH-primed recipients. In any given experiment, up to 10% of B10 mice can be expected to mount significant secondary responses to aqueous HEL challenge following priming by HEL in FCA (16). It was possible that the priming effect demonstrated herein was merely an artifact arising from the inclusion of lymphocytes from such a "responder" in the pooled spleen cell preparations which were utilized for these experiments. Accordingly an experiment involving one-to-one transfers of spleen cells from individual HEL/FCA-primed donors into individual KLH-primed recipients was carried out. Additionally, equal aliquots from each of these individual donor's cells were pooled and apportioned among a second group of recipients. All of the recipients were then challenged with HEL–KLH and their anti-HEL responses were assessed. As can be seen in Fig. 4, although pooling certainly dampened the overall variability of the response, two-thirds of the recipients in the individual transfer group were clearly within a range which we would consider to be high responder. Since this result could be obtained only if each donor contained primed cells, it seems unlikely that the experiments with pooled cells were skewed by the presence of a rare high responder cell donor.

Concomitant challenge with free HEL does not affect the response of HEL–KLH. In view of the finding that B10 mice which had been

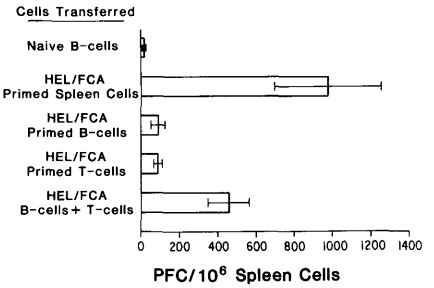

FIG. 3. Response to HEL–KLH of KLH-primed recipients of HEL/FCA-primed lymphocyte subpopulations. A large group of C57BL/10 mice were primed with 100 μg HEL in FCA and rested for 1 month, and their spleen cells were harvested. One portion of the whole spleen population was set aside and the remaining cells were fractionated into the T- and B-cell subpopulations as described. Naive B cells (saline/FCA) were similarly derived. These populations were then transferred alone or admixed, as indicated, into 600 R X-irradiated recipients which had been primed 1 month earlier with 100 μg KLH in FCA. All recipients were then challenged with 100 μg HEL–KLH and their HEL-specific PFC responses were assessed 6 days later. Limit bars represent standard error of the mean.

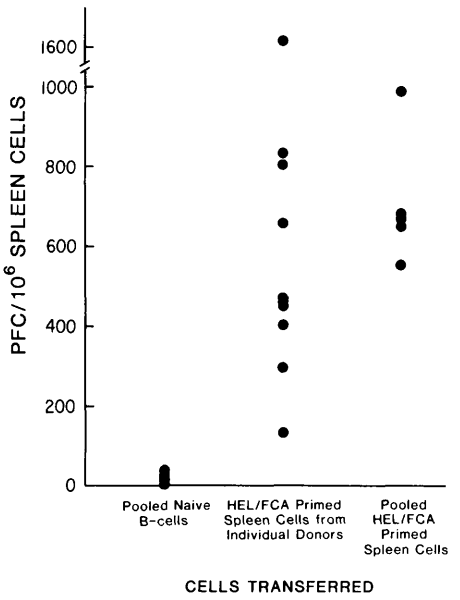

FIG. 4. Response to HEL–KLH of KLH-primed recipients of HEL/FCA-primed spleen cells transferred from individual donors. Each dot represents the response of one recipient. A group of 10 C57BL/10 mice was primed with 100 μg HEL in FCA. One month later individual spleen cell preparations were derived from each mouse. Portions from each individual preparation were pooled and distributed among five recipients. The remainder of each individual preparation was then grafted into a separate individual recipient. A third group of recipients received pooled, naive (Saline/FCA-primed) spleen cells. All recipients had been primed with 100 μg KLH 1 month earlier and were irradiated with 600 R on the day of transfer. Following the transfer the animals were challenged with 100 μg HEL–KLH and their HEL-specific PFC responses were assessed 6 days later.

concomitantly primed with KLH and HEL were able to respond to HEL–KLH (Fig. 1), we wondered whether the conjugation to KLH might have altered the ability of HEL to activate suppression. Accordingly we set up an experiment to assess the effect of incorporating native HEL into the challenge. KLH-primed B10 mice were irradiated with 600 R, grafted with spleen cells from HEL-primed B10 donors, and split into four groups. One group was challenged with HEL alone, the second with a mixture of HEL and KLH, the third with a mixture of HEL and the HEL–KLH conjugate, and the fourth with the HEL–KLH conjugate alone. As can be seen in Fig. 5, incorporation of free HEL into the HEL–KLH challenge had no effect on the anti-HEL response to the conjugate.

Discussion. The C57BL/10 mouse exhibits a complex, genetically determined defect in its ability to respond to HEL. When immunized under conditions that elicit strong splenic anti-HEL PFC responses in mice of most strains, B10 mice respond poorly if at all. Nonresponsiveness to HEL has been shown to be a recessive trait linked to the H-2^b and H-2^s haplotypes, although non-MHC effects have also been documented (16, 17). The defect has in part been attributed to the induction of an HEL-specific suppressor cell circuit in these animals (7, 8). This circuit has been shown to involve an idiotype-specific suppressor–inducer T cell and an HEL-specific suppressor–effector T cell (18, 19). These cells notwithstanding, these animals can generate primary anti-HEL PFC in both spleen and peripheral lymph nodes when immunized by the appropriate means (8, 20). This dichotomy has been attributed to both positional and temporal asychrony in the induction of the suppressor circuit (19, 20).

Our initial goal in undertaking the experiments reported here was to develop a new system to study the activity of HEL-specific suppressor cells *in vivo*. Central to this goal was the utilization of an HEL–KLH conjugate to focus KLH-specific help onto HEL-specific B

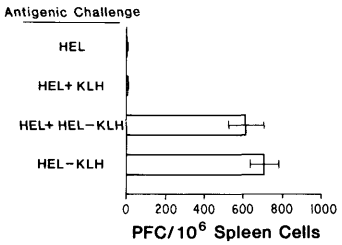

FIG. 5. Response to various antigen combinations of KLH-primed recipients of HEL/FCA-primed spleen cells. A large group of C57BL/10 mice were primed with 100 μg HEL in FCA and rested for 1 month, and their spleen cells were harvested. A second group was primed with 100 μg KLH in FCA, rested for 1 month, and then irradiated with 600 R. The HEL-primed spleen cells were then grafted into these recipients. The animals were then split into four groups, each group receiving a different challenge as indicated (100 μg of each protein was given for the combinations). The HEL-specific PFC responses of these animals were measured 6 days later. Limit bars represent standard error of the mean.

cells, thus circumventing any need for HEL specific helper cells in the nonresponder mice (8, 21). The experiment presented in Table I demonstrates that such a conjugate functions quite effectively in this way in known responder animals. To demonstrate the same effect in nonresponder B10 mice, we derived HEL-primed B cells from animals which had been immunized with HEL complexed to LPS, a reagent which previous studies had suggested might be capable of priming B cells in a nonresponder system (15, 22). That this was the case is demonstrated by the experiment presented in Table II.

Having demonstrated the hapten-carrier character of our HEL–KLH conjugate, we next set up an experiment to assess HEL-specific suppression in the B10 animals. Groups of these animals were primed with HEL alone, with KLH alone, or with a mixture HEL and KLH, and subsequently challenged with the HEL–KLH conjugate. Our expectation was that primed KLH-specific helper cells would render the animals capable of responding to the HEL portion of the conjugate, whereas simultaneous preimmunization with HEL would induce suppression and diminish this anti-HEL response. The KLH priming did indeed endow the animals with the ability to respond to the HEL portion of the conjugate, but concomitant priming with HEL enhanced, rather than diminished, this response. Thus, not only was suppression not evident, but the preexposure to HEL actually seemed to have a positive effect. Moreover, since immunization with neither KLH nor any of the multiple antigens present in FCA led to this enhancement it was clearly HEL specific (Fig. 1).

Others have reported that primary splenic anti-HEL PFC responses could be elicited in B10 mice by an HEL–burro erythrocyte conjugate (8). This response was suppressed if the mice were first immunized with a high dose of HEL several weeks prior to injection of the conjugate. Those investigators demonstrated *in vitro* that the suppression was specific for HEL and mediated by I–J^b-bearing T cells. These previous observations clearly differ markedly from the results of the experiments described here. However, it should be noted that each set of experiments dealt with a different phase of the immune response. Thus, we attempted to suppress a secondary response that was dependent upon the participation of carrier specific cells that had been primed under the influence of FCA. On the other hand, the previous investigators utilized HEL-primed cells to suppress a primary response elicited in the absence of adjuvant. The conditions under which the burro erythrocyte experiments were conducted may have been predisposed to suppression, whereas those employed in our experiments may have favored help. In order to assess the latter possibility we set up a series of experiments designed to define the underlying cellular basis of the observed enhancement. The first of these involved determining whether the enhancement could be adoptively transferred and if so, whether it resided in the T cell or B cell population. The results of this experiment, presented in Fig. 2, suggested rather strongly that some form of helper cell priming was taking place in the HEL/FCA immunized B10 nonresponder. This led us to wonder whether we might be able to detect B cell priming as well. As demonstrated in Fig. 3, this apparently does occur. Clearly, therefore, these animals exhibited some form of specific priming at both cellular levels.

There existed a possible trivial explanation for these results. Since these experiments involved the transfer of cells which had been pooled from multiple donors, it seemed conceivable that the priming effect we observed resulted from the inclusion of cells from the 10% of B10 animals which would be expected to respond anyway. However, this was not the case, as is demonstrated by the experiment presented in Fig. 4.

As pointed out, our initial goal in undertaking these experiments was to develop a system in which we could study the B10 HEL-specific suppressor cells *in vivo*. In previous studies, a suppressive epitope was identified on HEL. This epitope is localized at the surface of the molecule near the N-terminus, and its expression is thought to be dependent upon the presence of phenylalanine at amino acid residue No. 3 (23). Removal of an N-terminal tripeptide from HEL does indeed eliminate its ability to induce suppression in these mice (24). In view of the fact that our experiments with the HEL–KLH conjugate failed to detect suppression, we sought to determine if the procedure used for conjugating the HEL to

KLH might have rendered the suppressive epitope unrecognizable by the suppressor cells. To accomplish this we asked whether native HEL, if incorporated into the challenge along with the conjugate, would have any effect on the response to the latter. The expectation here was that the unconjugated lysozyme would activate HEL specific suppressor cells. However, as demonstrated in Fig. 5, no suppressive effect was observed. Although it is possible that one or more components of the suppressor circuit were lost or rendered inoperable as a consequence of the procedures involved in the adoptive cell transfers, we consider this unlikely in view of the absolute lack of HEL-specific PFC generated by recipients of HEL-primed spleen cells when challenged with HEL alone (Fig. 5). These animals would have been expected to respond had our experimental manipulations functionally altered the suppressor mechanism and tipped the regulatory balance in favor of help.

As pointed out earlier, following primary immunization with HEL coupled to appropriate carriers, C57BL/10 mice mount anti-HEL responses which are indistinguishable from those of responder strains (8, 15, 20). Indeed, it was these observations which led us to test HEL–LPS-primed B10 mice as a potential source of primed B cells for the experiment in Fig. 2. Such cells clearly were present in those animals and, in retrospect, one must assume that primed T cells were also present.

In summary therefore, previous studies and the present findings point to a system in which a primary antibody response can be mounted and in which both B and T cells can be primed, yet a secondary antibody response can only be induced if some form of "exogenous" help is supplied. The simplistic view, that the primed helper cells are prevented from acting by the suppressor cell circuit, is inconsistent with the finding that these cells are active in the presence of exogenous help. Thus, if suppressor cells are responsible for the lack of secondary responsiveness to HEL alone, their mechanism of action is clearly subject to circumvention. One model which is consistent with this idea is based on a postulated requirement for two helper cells in the B10 secondary response to HEL. In at least one experimental system it has been demonstrated that although IgM production to T-dependent antigens can be induced by a single helper-T-cell subset, a second, apparently idiotype-specific helper T cell subset was required for IgG secretion (25). Both idiotype-specific and carrier-specific helper T cells have been implicated in anti-HEL responses in B10.A mice (26), and it may be that both subsets of helper T cells are required for the induction of secondary anti-HEL responses in nonresponder B10 mice. If one of these helper cell populations were suppressed in the primed B10 animal, then HEL alone would not be expected to elicit a response. However, coupling the HEL to KLH would allow KLH-specific helper cells to fill the void. This of course assumes that B-cell priming could take place in the absence of this second helper population, which is not inconsistent with the above described requirement of only one helper cell for IgM responsiveness (25). Alternatively, the previously described asynchrony between helper and suppressor induction (19, 20) may allow a second helper to be originally induced and only subsequently eliminated.

An alternative explanation of our findings, one which does not invoke suppression, is suggested by previous studies which demonstrate a defect in antigen presentation in this system (9, 10, 27). In those experiments, anti-HEL PFC responses could be obtained when nonresponder spleen cells were presented with the antigen by responder antigen-presenting cells, whereas nonresponder antigen-presenting cells were unable to effectively cooperate with responder nonadherent spleen cells. As pointed out above, nonresponder C57BL/10 mice can nevertheless mount pronounced primary anti-HEL responses when the antigen is administered with a strong adjuvant (15, 22). This suggests that the antigen presentation defect can be circumvented. Thus, the present results could be explained by postulating that during primary immunization the antigen presentation defect is circumvented by the use of FCA and that during secondary immunization the defect is circumvented due to the utilization of the KLH carrier. Lacking the latter, the defect would not be circumvented and thus HEL alone would not elicit a secondary response even though primed lymphocytes were present. Experiments designed to address both of the above possibilities are currently in progress.

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