Single-cell studies on hapten-specific B cells: Response to T-cell-dependent antigens

(helper T cells/hapten-gelatin fractionation/bystander stimulation/B-cell subsets)

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ABSTRACT The effectiveness of the hapten-gelatin antigen-affinity fractionation technique for selection of hapten-specific B cells activatable by “T-cell-dependent” (TD) stimuli was assessed. Normal adult murine spleen cells were fractionated on fluorescein (Flu)-gelatin layers and the adherent cells were cultured singly or in small numbers with various sources of syngeneic keyhole limpet hemocyanin (KLH)-primed T lymphocytes. Conditions were defined under which the addition of Flu-KLH caused optimal clonal proliferation and differentiation of B cells into anti-Flu directly hemolytic plaque-forming cells (pfc). It was found that 3–5% of the Flu-specific B cells could be activated, versus 1 in 5000 unfractionated spleen cells. The mean enrichment factor for fractionation was 179, almost identical to that seen when the stimulus is “T-cell-independent” (TI), showing that the method is capable of isolating B cells responsive to antigenic stimuli requiring specific T-cell help. Efforts were made to determine whether TD B cells constituted a separate population from TI B cells by determining clone frequencies using Flu-KLH, the TI antigen Flu-polymerized flagellin (Flu-POL), or a mixture of both for stimulation. With Flu-POL alone and with the mixed stimulus 2–3 times more pfc clones were produced than with Flu-KLH, yet evidence for separate B-cell subsets was not obtained because of strong “bystander” stimulation due to the presence of the carrier-primed T cells in a confined volume of 10 µl.

The activation of B lymphocytes by antigen in tissue culture, leading to division and differentiation to antibody-producing cells, is influenced by many factors. Amongst these is the dependence of the response to the antigenic stimulus upon help from major histocompatibility complex (MHC)-restricted, antigen-specific T cells (1–3); the presence of macrophages or other accessory cells (4, 5); and the influence of non-MHC-restricted, antigen-nonspecific factors that promote B-cell growth, differentiation, or both (6–11). Our understanding of the activation process has been enlarged through the use of B cells bearing receptors for the particular epitope in question (12–18). Recently, methods have been developed that allow one single hapten-specific B cell, in the absence of any accessory or feeder cells, to respond to certain “T-cell-independent” (TI) triggering stimuli, including mitogens and antigens (10, 11, 19, 20). These have highlighted the great importance of T-cell-derived, antigen-nonspecific factors in the process and have allowed a reclassification of TI antigens on the basis of their dependence upon them for triggering (19, 20). Elegant cloning methods have also been developed that allow B cells to respond to “T-cell-dependent” (TD) antigens (21–23). However, only a few investigators have used isolated hapten-specific B cells as the responder population, and in most instances, only low cloning efficiencies were obtained (15, 16, 24). Given the considerable antigen-nonspecific influences that T cells can have on B cells, including the production of growth and differentiation factors and general support to the culture through “filler” cell effects (25), it seems desirable to develop a system in which one single B cell is the target and in which the minimal possible number of antigen-specific carrier-activated T cells provide the help. Only with the use of refined cloning methods of high efficiency will it be possible to address the question as to whether TD and TI B cells are phenotypically distinct populations (26–28).

This paper is a step in the direction of more refined cloning methods. It shows that the Haas and Layton technique (12) is just as effective in selecting for cells responding to TD stimuli. It differs from most recent work in avoiding either x-irradiation of helper T cells or the use of long-term cloned helper T-cell lines (16, 24). While it has demonstrated a moderately high cloning efficiency with TD stimulation, it has not resolved the issue of separate TI and TD B cells, because of operational constraints imposed by increments in cloning efficiency (“bystander” effects) that T-cell-derived lymphokines can have on B cells responding to TI antigens.

MATERIALS AND METHODS

Mice and Cell Suspensions. Specific pathogen-free inbred BALB/c An. Bradley Wehi mice were used at 8–10 weeks of age. Spleen cell suspensions were prepared as described (13).

Antigens. The hapten fluorescein (Flu) was conjugated onto keyhole limpet hemocyanin (KLH; Calbiochem) and onto polymerized flagellin (POL) as described (14). A Flu, sKLH and a Flu-POL conjugate were used. Nonconjugated KLH and 4-hydroxy-3-iodo-5-nitrophenylacetic acid (Nip)-conjugated KLH (Nip-KLH) were used where indicated.

Preparation of Flu-Binding Splenic B Cells. Spleen cells were fractionated on thin layers of hapten-gelatin as described (12–14). Adherent antigen was removed from the recovered binding cells prior to culture with collagenase treatment. This method yields populations of cells that are 97% B cells and approximately 70% Flu binding and about 200-fold enriched for in vitro reactivity to haptenated POL (14, 25).

Preparation of Helper T-Cell Populations. Donors of carrier-primed T cells were immunized by intraperitoneal administration of 200 µg of KLH in alum in the presence of 4 × 10⁹ Bordetella pertussis organisms, boosted 4 weeks later with 50 µg of soluble KLH intraperitoneally, and sacrificed 3 days after boosting. Splenic T-cell populations were prepared by two passages over nylon wool columns as described (29). This population will be referred to as nylon-wool-nonadherent splenic T cells. Where indicated, these T cells were further enriched for KLH reactivity by their adherence to KLH-pulsed monolayers of peritoneal exudate cells.

Abbreviations: Flu, fluorescein; KLH, keyhole limpet hemocyanin; MHC, major histocompatibility complex; Nip, 4-hydroxy-3-iodo-5-nitrophenylacetic acid; pfc, plaque-forming cell; POL, polymerized flagellin; TD, T-cell-dependent; TI, T-cell-independent.
cells, using the method of Langdon et al. (30). Short-term in vitro propagated T-cell lines were prepared essentially as described by Augustin et al. (31). Briefly, $25 \times 10^6$ spleen cells from KLH-primed and boosted donors were cultured in 5 ml of RPMI medium containing 5% (vol/vol) fetal calf serum, 50 μM 2-mercaptoethanol, and KLH at 40 μg/ml in a 25-cm$^3$ flask in the upright position. At weekly intervals thereafter, viable cells were selected by centrifugation over Ficoll-PAque (Pharmacia) and recultured with KLH in the presence of irradiated syngeneic spleen cells. Viable cells were selected for use as T-helper cells after two to four passages.

Microculture System and Assay for Antibody Formation. Microcultures were set up in 60-well, 10-μl Terasaki trays as described (19, 20, 25), using RPMI 1640 medium supplemented with 5% (vol/vol) fetal calf serum and 100 mM 2-mercaptoethanol. Flu-KLH, KLH, and Nip-KLH were used at 50 ng/ml and Flu-POL at 100 ng/ml. Unfractionated spleen cells or Flu-gelatin-binding B cells were cultured in the presence of splenic T cells from KLH-primed donors as indicated above. Where indicated, $5 \times 10^4$ irradiated [2000 rads (20 grays)] syngeneic spleen cells were added. Cultures were individually assayed for anti-Flu plaque-forming cells (pfc) as described (32).

Statistics. The frequency of clonal precursors was determined as described either by Poissonian analysis (33) or by a method of simultaneously calculating maximum likelihood estimators (34).

RESULTS

Helper Activity of Splenic T Cells from KLH-Primed Donors. Prior to assessing the responsiveness of Flu-gelatin-binding splenic B cells to a TD stimulus, the efficacy of using various sources of helper T cells was investigated. In the first instance, the capacity of nylon-wool-nonadherent splenic T cells from KLH-primed and boosted donors after either further fractionation on a monolayer of KLH-pulsed macrophages or repassage over nylon wool to provide help for a Flu-KLH-elicited response from unprimed unfractionated syngeneic spleen cells was assessed. As shown in Fig. 1, a significant anti-Flu pfc response was generated with as few as 500 macrophage-adherent T cells, the response being maximal with around 2000 T cells (Fig. 1a), whereas around 20,000–40,000 nylon-wool-nonadherent T cells was found to be optimal (Fig. 1b). The results with low cell numbers indicated that the macrophage adherence procedure yielded a population enriched about 10-fold for helper activity. However, as only low numbers of cells were recovered with this technique it was not considered practicable for large numbers of replicate cultures as required for limiting-dilution analysis. The responses elicited in the absence of antigen were similar to the two helper populations.

Helper Capacity of Propagated KLH-Primed T Cells in Vitro. The helper capacity of short-term in vitro propagated splenic T cells from KLH-primed and boosted donors was next investigated. Fig. 2 shows the anti-Flu pfc responses elicited by Flu-KLH from unfractionated spleen cells when cultured with various numbers of such T cells. Significant responses were obtained with as few as 250 T cells, with the maximal pfc response being reached with 750–1000 T cells. These findings are somewhat similar to those reported by others, using cloned T-cell lines (35).

The helper activity exhibited by these T cells was thus about 2–3 times more efficient than that of splenic T cells selected by antigen binding (Fig. 1a) and about 20 times more efficient than nylon-wool-nonadherent splenic T cells (Fig. 1b). However, the response generated in the absence of Flu-KLH was higher than seen with the noncultured splenic T cells.

Frequency of Flu-KLH-Responsive Splenic Cells. Limiting-dilution analyses were performed to determine the frequency of anti-Flu pfc clonal precursors amongst unfractionated spleen cells from unprimed donors when stimulated with Flu-KLH in the presence of nonlimiting numbers of nylon-wool-nonadherent splenic T cells both before and after prop-
agitation in vitro. As shown in Fig. 3, both T-cell populations supported a linear response. The frequency of clonal pfc precursors was higher in the presence of in vitro propagated T cells (1 in 3600 spleen cells) (Fig. 3b) than in vivo derived T cells (1 in 7000 spleen cells) (Fig. 3a). These frequency values fall within the range of the values published for a TI-elicited response (25). Short-term in vitro propagated helper T cells were selected for further studies on the basis of their higher efficiency.

Response of Isolated Flu-Specific B Cells to Flu-KLH in Vitro. The frequency of anti-Flu pfc clone precursors amongst Flu-gelatin-binding B cells was next determined by using the limiting-dilution analysis. Short-term in vitro propagated helper T cells were used (1500 per culture) and syngeneic irradiated spleen cells (5 x 10^3 per culture) were added to provide antigen-presenting cells to ensure that responder B cells were the only limiting entity in the assay. Fig. 4 shows the results of one such analysis, in which 1 in 20 Flu-specific B cells was shown to form an anti-Flu pfc clone in response to Flu-KLH in the presence of short-term in vitro propagated helper T cells. As with unfractonated spleen, this represents a value similar to that reported for a TI stimulus (14, 25), and thus the mean enrichment value obtained by the fractionation procedure is also similar.

Antigen-Specific and Nonspecific Helper Activity of T-Cell Lines. Shown in Table 1 are the frequency values obtained in a series of experiments in which either unfractonated spleen or Flu-specific B cells were stimulated with either Flu-KLH or Nip-KLH (as a “carrier-effect” control) in the presence of 1500 in vitro propagated helper T cells for 4 days prior to assay for anti-Flu pfc clones. The bystander or nonspecific response generated in Nip-KLH-containing cultures was approximately one-third of the response generated in the presence of Flu-KLH. No marked difference was seen whether Nip-KLH, KLH, or no antigen was present (data not shown). The responses generated in the presence of Flu-KLH were statistically different from those with Nip-KLH (0.0025 > P > 0.0005) with both B-cell sources. This bystander response was shown to be non-MHC-restricted (data not shown).

Responsiveness of Flu-Specific B Cells to TD and TI Stimuli. Having established the frequency of TD-responsive cells amongst Flu-gelatin-binding B cells, we next investigated whether the responsiveness of this population to TD and to TI antigens was additive. Limiting-dilution analyses were performed using Flu-KLH as a TD antigen and Flu-POL as a TI antigen. As shown in Table 2, the response elicited by Flu-KLH alone was below that of Flu-POL either alone or in the presence of Nip-KLH. The Flu-POL response is somewhat higher than the 3–5% reported for Flu-POL in the presence of thymus filler cells (14, 25) and is probably increased as a result of a lymphokine-mediated filler cell effect (25). In a single experiment the intentional addition of EL4-conditioned medium containing B-cell growth and differentiation factors (11) slightly increased the Flu-POL response from 8.0% to 11.3% when T cells were present, whereas with Flu-POL and EL4-conditioned medium alone with no T cells present, the response was 4.1%. The frequency of Flu-KLH-reactive cells in this particular experiment was 3.4%.

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**Fig. 2.** Helper capacity of T cells from short-term in vitro propagated T-cell lines. Anti-Flu pfc responses generated by nonprimed unfractionated syngeneic spleen cells when stimulated by Flu-KLH (○) or KLH (×) in the presence of various numbers of T cells.

**Fig. 3.** Limiting-dilution analysis of the frequency of splenic B cells responsive to Flu-KLH when cultured in the presence of either 5 x 10^3 nylon-wool-nonadherent splenic T cells (a) or 2500 short-term in vitro propagated helper T cells (b), both derived from KLH-primed donors. The frequency of anti-Flu pfc precursors was calculated to be 143 x 10^-6 (95% confidence limits 114–171, indicated by the broken lines) for a and 278 x 10^-6 (219–337) for b. In the presence of any added antigen, frequency values of 41 x 10^-6 (30–51) for a and 140 x 10^-6 (92–162) for b were obtained.
FIG. 4. Limiting-dilution analysis of the frequency of Flu-KLH-responsive cells amongst Flu-gelatin-binding B cells when cultured in the presence of 1500 short-term in vitro propagated helper T cells and 5 x 10^4 syngeneic irradiated spleen cells. The frequency of anti-Flu pfc precursors was calculated to be 4.92 x 10^-2 (3.83-6.11). In the presence of Nip-KLH, a frequency value of 2.14 x 10^-2 (1.50-2.78) was obtained.

DISCUSSION
This paper describes a method that allows isolated hapten-specific B cells to respond to a TD antigen with a cloning efficiency of 3-5%, approximately similar to that which we deemed satisfactory for TI stimulation for many years, before our recent descriptions of more efficient methods. It is satisfying to note that the hapten-gelatin fractionation technology (12-14) is just as effective for "TD-responsive B cells" as for B cells responding to TI stimuli if such separable subsets exist. The enrichment factor of 179 here reported is almost identical to that of 176 recently reported (25) in a TI system. This suggests that if virgin TI- and TD-responsive B cells are distinct subsets, they must exhibit approximately similar spectra of antigen-binding avidities.

We compared the helper capacity of three types of primed and reactivated carrier-specific T cells. Carrier-primed and boosted spleen cells passed over nylon wool columns, but not otherwise enriched or treated, proved only moderately effective, and 20,000-40,000 cells were required to achieve maximal help to B cells, as reported in earlier work by Waldman et al. (23). T cells enriched for carrier specificity through binding to and removal from carrier antigen-bearing macrophage monolayers worked 10- to 20-fold more effectively, providing a useful validation of the method of Landdon et al. (30).

Table 2. Comparative cloning efficiencies of hapten-specific B cells with TD and TI stimuli

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<tr>
<th>Stimulus*</th>
<th>Anti-Flu pfc clone frequency</th>
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<tr>
<td>Flu-KLH</td>
<td>333 ± 46 (X 10^-4)</td>
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<tr>
<td>Nip-KLH</td>
<td>170 ± 57 (X 10^-4)</td>
</tr>
<tr>
<td>Flu-POL + Nip-KLH</td>
<td>863 ± 20 (X 10^-4)</td>
</tr>
<tr>
<td>Flu-POL + Flu-KLH</td>
<td>1020 ± 52 (X 10^-4)</td>
</tr>
<tr>
<td>Flu-POL</td>
<td>820† (X 10^-4)</td>
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</table>

Flu-specific B cells were cultured at limit dilution in the presence of helper T cells and stimulated with the antigens as indicated. Results are mean ± SEM of three separate experiments.
*Flu-KLH or Nip-KLH at 50 ng/ml, Flu-POL at 100 ng/ml.
†Results of two experiments only.

However, the most effective and convenient sources of helper T cells were populations derived from carrier-primed spleen cells after maintenance in tissue culture with carrier antigen and x-irradiated spleen cells for 2-4 weeks prior to use. These populations provided substantial help when as few as 250 cells were added, and 750-1500 cells gave maximal effects, numbers comparable to those cited for helper cell lines (35).

Given that these noncloned, short-term in vitro propagated T cell populations were far from homogeneous either in their morphology or in their specificity, this suggests both that the efficiency of cloned lines on a cell-for-cell basis may not be ideal and that further purification of the short-term, physiologically normal T cells should prove rewarding. For example, the T cells for the preparation of lines could be "preselected" on KLH-bearing macrophages, treated to remove Lyt 2+ suppressor cells, or both. Direct quantitative comparison with the new conventional long-term helper lines would then be particularly interesting.

The biggest problem we have encountered in this work is the activation induced amongst B cells in the absence of the correct hapten-carrier antigen. The activated T cells were probably secreting B-cell-active lymphokines and differentiation factors while in bulk culture (36), and they have continued to do so in the clonal 10-μl microcultures supported by the KLH carried over. This lymphokine secretion would have been augmented by the addition of either Flu-KLH or the control antigen Nip-KLH. In the presence of a sufficient density of fitter cells, such lymphokines can induce polyclonal B-cell activation (25, 36), and it is not established whether the targets on which they act are TD- or TI-responsive B cells. When Flu-POL and Nip-KLH were used as stimuli, the number of B cells responding was no greater than for Flu-POL stimulus acting alone. This suggests that the lymphokine level within cultures stimulated by Flu-POL alone was already adequate, probably because of continued secretion by the activated T cells. Thus, the response was not significantly raised by additional carrier stimulation of the T cells. The Flu-KLH response was significantly higher than the Nip-KLH response (Table 1), and MHC-compatible T-cell sources were significantly more effective than MHC-incompatible sources (data not shown), showing that an antigen-specific MHC-restricted response was being induced. The ratio of this specific response to bystander stimulation could be improved by reducing the T-cell number to 250 (Fig. 2), but this also resulted in fewer B-cell clones being triggered (data not shown). It is possible that B-cell clones arising under conditions of suboptimal help are of higher affinity, and this would be worth testing directly. It is also possible that higher clone frequencies may be obtained with hapten-primed B cells. Whilst we are far from satisfied with the cloning efficiency in the TD cloning system used, both the clone frequency and the total number of pfc per input B cells are far higher than reported in the literature (16, 24).

In previous work (37) we have questioned the convention-

Table 1. Antigen-specific and nonspecific helper activity of T-cell lines

<table>
<thead>
<tr>
<th>Stimulus*</th>
<th>Unfractionated spleen cells</th>
<th>Flu-gelatin-binding B cells</th>
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<tr>
<td>Flu-KLH</td>
<td>204 ± 74 (X 10^-4)</td>
<td>365 ± 29 (X 10^-4)</td>
</tr>
<tr>
<td>Nip-KLH</td>
<td>61 ± 5 (X 10^-4)</td>
<td>181 ± 42 (X 10^-4)</td>
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Responder B cells were stimulated at limit dilution in the presence of 1500 cells from short-term helper T-cell lines and 5 x 10^4 irradiated spleen cells. Data represent mean ± SEM of pooled data from 4-13 individual directly comparative experiments.
*Flu-KLH or Nip-KLH was present at 50 ng/ml.
al wisdom suggesting that the Lyb 5 marker is useful for discriminating T1- from TD-responsive B cells. We have also found the presence or absence of IgD to be unhelpful in this regard (38, 39). In the present study, we attempted to use single or mixed stimulation of hapten-specific B cells with T1 and TD antigens with analysis at the clonal level to approach the question. The results (Table 2) were not inconsistent with the possibility that the responding B-cell populations were distinct entities, but the number of B cells stimulated by T1 antigen and bystander help was too close to the number stimulated by both T1 and TD antigens acting together for us to have confidence in the distinction. Further optimization and refinement of the TD cloning system should allow the problem to be addressed in future studies in a definitive manner.

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