extracts was a 15-m (0.53-μm film, 1.0-mm inner diameter) Carbowax (Quadrex). Columns were operated at 60°C for 2 min, and then the temperature was programmed at 30°C/min to 160°C. Identification of attractive compounds was based on electron impact and chemical ionization (methane) mass spectroscopy with sample introduction via capillary GC. Fourier transform infrared spectra were obtained on the active compounds. Proton nuclear magnetic resonance spectra (300 MHz) was obtained on natural linalool. The chirality of the *d*-linalool was determined by capillary GC analysis [with a chiral val capillary column (Chrompack, Inc.)] of the diastereomers obtained with (*R*)-phenyl isocyanate, according to the method of E. M. Gaydou and R. P. Randriamihariso [*J. Chromatogr.* **396**, 3781 (1987)]. The *d*-linalool for bioassays was obtained from coriander seeds. The oil obtained was purified by preparative GC and the material (>99% purity) had an enantiomeric purity of 95% dextrorotatory and 5% levorotatory. The *m*- and *p*-cresol (Aldrich) were purified to >99% purity before use.

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The Minimal Number of Class II MHC–Antigen Complexes Needed for T Cell Activation

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Major histocompatibility complex (MHC) molecules are exposed to large quantities of self and nonself antigens. It is not known what fraction of MHC molecules needs to be occupied by antigen to induce a T cell response. A quantitative study of naturally processed antigen indicated that T cells could be activated when only 0.03 percent of the total I-E^d purified from antigen-presenting cells (APCs) was occupied with antigen. B cells and macrophages processed hen egg lysozyme (HEL) with different efficiencies, but similar degrees of occupancy were required for T cell stimulation. Higher occupancy was needed for I-E^d–transfected L cells, possibly reflecting the requirement for other accessory molecules for efficient APC–T cell interaction.

THE HEL PEPTIDE THAT CORRESPONDS to the I-E^d–restricted determinant, HEL(107–116), can be isolated from naturally processed antigen (*1*). When HEL concentrations of 1 mg/ml are added to APCs, a substantial fraction (10 to 40%) of I-E^d molecules is occupied by HEL-derived antigen fragments (*1*). We have conducted experiments to estimate the minimal number of MHC-antigen complexes necessary to induce T cell activation. Large numbers (~10^9) of the B lymphoma, 2PK3 (*2*), were pulsed with various concentrations of HEL. After 24 hours, cell samples were washed, and their capacity to activate the I-E^d–restricted, HEL(107–116)–specific, T cell hybridoma, IE5, was assessed (*3*). Doses above 1 μg/ml gave an optimal response, and a dose of 0.5 μg/ml or less induced suboptimal interleukin-2 (IL-2) release from IE5 cells (Fig. 1). In parallel to these studies with intact APCs, I-E^d (including I-E^d–HEL peptide complexes) was purified by affinity chromatography (*1*) from each of the cell populations pulsed with the various HEL concentrations, and subsequently these preparations were inserted into planar membranes and used to stimulate IE5 cells (Fig. 2A). For HEL concentrations below 250 μg/ml, the relative potency of the different I-E^d preparations varied as a function of the HEL concentration used in the antigen pulse. To obtain a quantitative estimate of the fraction of I-E^d molecules that were occupied by the HEL(107–116) epitope in the various preparations, we also assessed the relative potency of HEL(107–116)–I-E^d complexes formed in vitro. At the same time, the concentration of antigen–I-E^d complexes formed was determined by Scatchard analysis (*1, 4*). In this way, IL-2 production could be related to the amount of HEL(107–116)–I-E^d complexes used in the T cell hybridoma assay (*1*) (Fig. 2B). Concentrations of 0.7 nM of HEL(107–116)–I-E^d complexes were required to stimulate IL-2 production at 40 U/ml from IE5 cells. About 230 nM of I-E^d purified from cells pulsed at the lowest concentration of HEL that gave stimulatory planar membranes (4 μg/ml) was necessary to achieve similar levels of IL-2 production. It can therefore be estimated that 0.7/230 (0.3%) of the I-E^d molecules purified from 2PK3 pulsed with HEL at 4 μg/ml were occupied by antigenic material. When similar calculations were performed for the other HEL concentrations used to pulse 2PK3 cells, a linear relation resulted (Fig. 2C). If linearity is assumed at low pulsing antigen concentrations, then an estimate of the percentage of

Table 1. Minimal number of I-E^d–antigen complexes required for T cell activation in various APCs. The numbers of I-E molecules per cell was determined by radiolabeling and immunoassay (*5*) with ^{125}I-labeled 14-4-4 monoclonal antibody to detect I-E^d and is stated as the average of two independent experiments. Experiment to experiment variability was contained within 40% of the mean. HEL dose is stated as micrograms per milliliter. Percent occupancy was determined from the capacity of I-E^d isolated from antigen-pulsed cells to stimulate IE5 cells in a planar membrane system, as described in Fig. 2. Average of two independent experiments. Occupancy levels for each cell type varied less than fourfold in replicate experiments.

Measure	APC types			
	2PK3	B cells	BMMϕ	CA36.2.1
Number of I-E molecules per cell	1.8×10^5	0.8×10^5	1.1×10^5	10^5
HEL dose required for suboptimal responses (320 U/ml)	0.3	2	256	32
Percent occupancy	0.03	0.14	0.25	4.6
Number of complexes	60	110	280	4600

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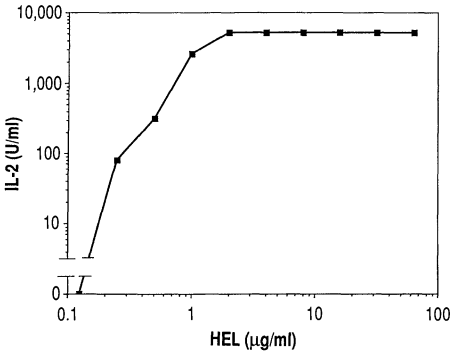

Fig. 1. Dose response for induction of IL-2 release in IE5 cells by native HEL. IE5 T cells (10^5) were cultured with 10^5 2PK3 B lymphoma cells in the presence of a range of HEL concentrations. After 24 hours, supernatants were assayed for IL-2 (*1*). One representative curve is shown. Antigen dose required for suboptimal (320 U/ml) response was, in the 12 experiments performed during the course of this study, 0.5 ± 0.25 μg/ml.

I-E^d occupied at 0.5 μg of HEL per milliliter (the concentration required to stimulate a suboptimal T cell response with the use of live APC) can be made. It can thus be calculated that as little as 0.03% occupancy is sufficient for T cell activation (Fig. 2C). Since the number of I-E^d molecules expressed on the surface of each 2PK3 cell, as quantitated by radiolabeling and an immunoassay (*5*), is 1.8×10^5 (Table 1), it can be estimated that as few as 60 I-E^d–antigen complexes may be sufficient to activate IE5 T cells.

We next studied the generalizability of the experiments with the 2PK3 B lymphoma by determining the relative efficacy of two normal APC types, lipopolysaccharide- and dextran sulfate–activated B cell blasts (*5*) and γ-interferon–induced bone marrow–derived macrophages (BMMϕ) (*6*), and a third APC population, I-E^d–transfected L cells, CA36.2.1 (*7*) (Fig. 3). B cell blasts and 2PK3 cells were more efficient than BMMϕ and transfected L cells. More specifically, HEL (~0.3 μg/ml) was sufficient to induce production of IL-2 at 320 U/ml, in the case of 2PK3 cells, and HEL (2 μg/ml) could induce a similar response in the case of B cell blasts. By contrast, antigen concentrations of 32 μg/ml were required for the transfected L cells and 260 μg/ml for BMMϕ. These differences cannot be explained on the basis of differential I-E^d expression alone, because by radiolabeling and immunoassay (*5*) (Table 1), I-E^d expression on these APC types varied by no more than twofold.

We next determined whether these differential potencies were reflective of differences in the capacity to generate I-E^d–antigen complexes from the native molecules (processing differences) or rather reflected differences, even at similar occupancies, in the capacity to induce IL-2 release from IE5 T cells (presenting differences). The results, summarized in Table 1, were obtained from experiments in which the occupancy of MHC with specific antigen was analyzed as described above in detail for 2PK3 cells. To achieve the same suboptimal level of T cell stimulation, 2PK3 cells required the least antigen (0.3 μg/ml), B cells required somewhat more (2 μg/ml), but CA36.2.1 cells required 15 times the amount required for B cells, and BMMϕ required 100 times more than B cells (Table 1). However, when the number of antigen-MHC complexes required to stimulate T cells was calculated, BMMϕ and B cells were similarly efficient. Only a few hundred complexes on the surface of a macrophage or B cell could provide a sufficient stimulus for IE5 cells (Table 1). Finally, in contrast to BMMϕ, B cell blasts, and 2PK3 cells, the L cell transfectants required more antigen-MHC complexes (~4600) to achieve a similar degree of stimulation. Although the reason for this relative inefficiency is not known, the failure of L cells to utilize the adhesion between leukocyte function–associated antigen–1 and its ligands, intercellular adhesion molecules 1 or 2, may account, in part, for the inefficiency (*8*).

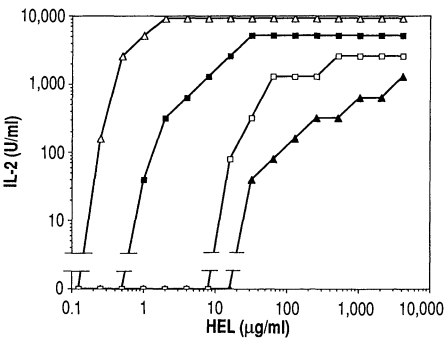

Fig. 3. Induction of IL-2 release by HEL in IE5 cells by different APC types (△, 2PK3; ■, B cell blasts; □, CA36.2.1; and ▲, BMMϕ). APCs (10^5) were cultured with 10^5 T cells and indicated doses of antigen. After 24 hours, supernatants were assayed for IL-2 (*1*). One of two independent titration experiments is shown. Variation from experiment to experiment in suboptimal HEL concentrations for the various APC types was no greater than twofold.

The relative inefficiency of macrophages to process HEL to immunologically relevant epitopes was surprising. These findings may reflect a much more active antigen catabolism in BMMϕ (*9*), compared to APCs of the B cell lineage. Whether this will be generalizable to other antigen epitopes and other macrophage populations, or is specific for this particular HEL epitope, is currently

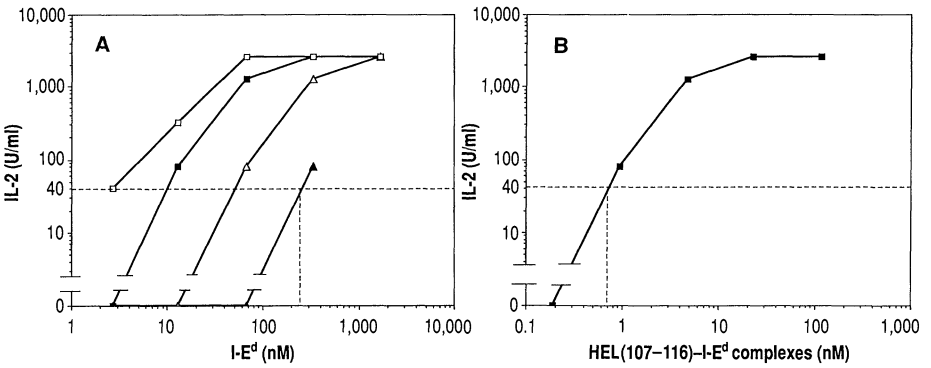

Fig. 2. Occupancy of I-E^d molecules purified from B lymphoma cells pulsed with different amounts of HEL. (**A**) I-E^d molecules affinity-purified from 2PK3 cells pulsed with various amounts of HEL (▲, 4 μg/ml; △, 16 μg/ml; ■, 64 μg/ml; and □, 256 μg/ml) were inserted into planar membranes and used to stimulate IL-2 release from IE5 T cells (*1*). (**B**) Generation of a standard curve, by testing various concentrations of in vitro preformed HEL(107–116)–I-E^d complexes in the same assay. (**C**) Estimation of the fraction of I-E^d molecules occupied by HEL-derived fragments in the various I-E^d preparations, as a function of the concentration of HEL used in the pulse. 2PK3 B lymphoma cells were pulsed for 24 hours with various concentrations of HEL, and I-E^d was purified from ~10^9 cells. I-E^d was inserted into planar membranes at different concentrations, and the total amount of I-E^d was kept constant by adding I-E^d purified from unpulsed 2PK3 B lymphoma cells (*1*). In parallel, I-E^d was incubated for 24 hours with a saturating concentration of synthetic HEL(107–116) peptide. I-E^d was recovered from free peptide by passage over a Sephadex G-50 column and inserted into planar membranes. The percentage of active I-E^d molecules was estimated by Scatchard analysis (*1, 4*). The amount of IL-2 released by two independent T cell cultures for each data point was measured. Identical IL-2 levels were detected in ≥95% of the cases.

under investigation.

The number of MHC-antigen complexes required to stimulate T cells is likely to vary not only with the APC source, but with the T cell population as well. The affinity of the T cell receptor for the antigen-MHC complexes is undoubtedly one important factor in determining the number of complexes required for T cell activation. The T cell hybridomas used in this and other studies have been very often selected for their high reactivity with antigen, and therefore the number of antigen-MHC complexes required to activate these cells may differ from the requirements for normal T cell activation.

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Adhesion of Human B Cells to Germinal Centers in Vitro Involves VLA-4 and INCAM-110

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Human B lymphocytes localize and differentiate within the microenvironment of lymphoid germinal centers. A frozen section binding assay was developed for the identification of those molecules involved in the adhesive interactions between B cells and lymphoid follicles. Activated human B cells and B cell lines were found to selectively adhere to germinal centers. The VLA-4 molecule on the lymphocyte and the adhesion molecule INCAM-110, expressed on follicular dendritic cells, supported this interaction. This cellular interaction model can be used for the study of how B cells differentiate.

THE GERMINAL CENTER OF THE LYMPHOID follicle is a specialized microenvironment that participates in the proliferation and differentiation of B lymphocytes. B lymphocytes enter lymphoid organs by first binding to high endothelial venules via specific adhesion receptors and subsequently migrating between the endothelial cells into the stroma (*1–4*). In the lymphoid organ they proliferate within the germinal center and then differentiate into either memory or immunoglobulin-producing cells (*5*). The major constituents of germinal centers are activated B cells, but follicular dendritic cells (FDC) as well as some macrophages and T cell subsets are also located there (*6, 7*). Within the germinal center, activated B cells are physically associated with the FDCs, but the precise functional interactions between B cells and other cellular components of the germinal center are unknown (*8*).

It is presently unknown how B cells localize within follicles or their germinal centers. Since the interaction of lymphocytes with high endothelial venules was characterized by preferential binding to endothelial cells in frozen tissue sections (*9*), we reasoned that a similar assay might be developed to show adhesion of B cells to follicles or their germinal centers. Highly purified human splenic B cells (>95% $CD20^+$) that were either unstimulated or cultured for 3 days with *Staphylococcus aureas* Cowan Strain I (SAC) were internally labeled with the fluorochrome sulfofluorescein diacetate (SFDA) to permit their visualization when bound to particular areas of tissue sections. The B cells cultured in SAC were determined to be activated by their increased cell size and expression of several B cell activation antigens (*10–12*). When activated B cells were incubated at 25°C on frozen sections of human tonsil, they adhered preferentially to germinal centers, as shown by combined fluorescence and bright-field microscopy (Fig. 1A and Table 1). Rare cells binding to extrafollicular areas, including microvessels, were also seen. Binding of SAC-stimulated cells to the germinal centers was essentially absent at 4°C. Unstimulated B cells showed little to no binding to germinal centers at either 25° or 4°C (Fig. 1B and Table 1). This result suggests that following activation, B cells undergo changes in cell surface antigens that facilitate their retention in germinal centers.

Considering the well-recognized adhesion between leukocytes and endothelial cells observed in frozen section binding assays, we examined whether the binding of B cells to germinal center regions could be explained on the basis of such an interaction. Sections were stained immunohistochemically with monoclonal antibody (MAb) to von Willebrand factor for identification of endothelial cells (Fig. 1C). Scattered capillaries were present within germinal centers, but their distribution was insufficient to account for the pattern and extent of B cell binding. This result indicates that activated B cells were adhering to structures other than endothelium within the germinal center.

Lymphoid cell lines with distinct cell surface phenotypic characteristics and endothelial binding properties have been useful in the identification of a number of homing receptors (*13, 14*). We examined expression

Table 1. Binding of resting and stimulated B cells to germinal centers. Resting and stimulated splenic B cells were used in the binding assay as described (Fig. 1) for 25 min at 25°C. Slides were independently examined blindly. Cells were counted on fluorescence photomicrographs, and germinal center areas were assessed on bright-field photomicrographs of the same sections with a Zeiss VideoPlan digitizing board. Splenic B cells from three individuals were examined for their binding to tonsillar sections from three different individuals and expressed as mean ± SEM number of cells bound per 0.1 mm^2 of germinal center area for three different tonsils.

Donors	Lymphocytes bound/0.1 mm^2	
	Resting B	Stimulated B
1	0.4 ± 0.2	235.0 ± 31.9
2	0.3 ± 0.3	212.9 ± 12.9
3	1.9 ± 0.5	145.7 ± 49.4

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