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Three Classes of Signalling Molecules on B-Cell Membranes

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The question of whether surface immunoglobulin and Ia molecules have a signalling function in helper T cell-dependent activation of B cells has been evaluated. Two sources of B cells have been used, one a purified population of hapten-binding B cells, the other a B-cell lymphoma, CH12, with known antigen specificity. Evidence is presented that both immunoglobulin and Ia molecules are receptors actively involved in the initial activation of resting B cells. Nevertheless, the requirements for ligand binding to either receptor can be bypassed under appropriate conditions, and the implications of this result for the function of these molecules is discussed. With respect to B-cell Ia, the authors present data that demonstrate two distinct functions of this molecule, one as a restricting element for T-cell activation, the second as a signalling receptor for B-cell excitation. On the CH12 surface, the I-A molecule fulfills the former function, but T-cell interactions with I-A fail to result in B-cell stimulation, suggesting that B-cell Ia may limit helper T cell-B cell interactions. We suggest that the binding of antigen surface immunoglobulin and binding of helper T-cell receptors to the appropriate Ia molecule(s) results in the activation of genes that encode for a third class of membrane B-cell receptors, those that bind B-cell stimulating factors.

Key words: helper T cell-B cell interactions, surface immunoglobulin, Ia molecules, membrane receptors, signal transducers

The requirements for helper T (Th) cell-dependent B-cell activation vary according to the activation state of the responding B cell [1-5]. B cells that have been activated recently and have not reverted to a resting state are stimulated to further proliferation and differentiation by antigen nonspecific cytokines. In contrast, resting B cells require both specific antigen and major histocompatibility complex (MHC) restricted interactions with carrier specific Th cells for activation. Cognate interac-

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tions between Th cells and resting B cells are facilitated by a hapten-carrier bridge [6]. The question of whether the B-cell surface immunoglobulin (sIg), B-cell-encoded Ia molecules, or both are receptors responsible for initiating entry of resting B cells into cell cycle following binding of the appropriate ligands (specific hapten and T-cell receptors, respectively) has been controversial and is the subject of this study.

The ability to evaluate the roles of sIg and Ia molecules in the activation of resting B cells requires that sIg-ligand binding be dissociated from Th-Ia interactions so that either can be achieved independently. This can be accomplished by taking advantage of the ability of B cells to present antigen to Th cells, irrespective of B-cell specificity as dictated by its sIg molecule [7,8]. The present study has used two sources of B cells, the first a B-cell lymphoma, representing a clonal population, and the second a population of normal B cells homogeneous with respect to hapten specificity. It is demonstrated that both sIg and MHC-encoded Ia molecules on the B-cell surface are functional receptors responsible for the activation of resting B cells, resulting in the expression of nonclonally distributed receptors for factors that promote B-cell growth and differentiation.

MATERIALS AND METHODS

Animals

(BALB/C × C57BL/6)F1 (CB6F1, H-$2^{d/b}$), BALB/c (BALB,H-2^{d}), C57BL/6 (B6, H-2^{b}), C3H/HeJ (H-2^{k}), B10.A(3R) (H-2^{i3}), B10.A(5R) (H-2^{i5}), and B10.A(2R) (H-2^{h2}) mice were purchased from Jackson Laboratory (Bar Harbor, ME), or bred in the animal colony at Duke University. B10.H-2^{a}H-4^{b}p/Wts ($2^{a}4^{b}$) mice were bred at the University of North Carolina. B10.A(3R) and B10.A(4R) (H-2^{h4}) kindly were provided by Dr. Jeffrey Frelinger, University of North Carolina. Individual loci within the H-2 complex relevant to this study are listed where appropriate in Results.

Helper T Cell Lines

Two types of Th cell lines were used in this study, and the preparation, maintenance, and properties of both have been published in detail. Alloreactive Th cell lines were enriched in primary and secondary mixed lymphocyte cultures (MLC) as described [9,10]. Keyhole limpet hemocyanin (KLH)-specific Th cell lines were established with T cells purified from draining lymph nodes of immunized mice [11]. F1 T cells were grown on parental antigen presenting cells (APC) in order to select for parent H-2-restricted T cells [11].

CH12 B Lymphoma Cells

The induction and characterization of the CH12 lymphoma have been described [12]. The sIg molecule of CH12 cells is an IgM, κ with specificity for sheep erythrocytes (SRBC). CH12 is maintained as an ascites tumor in $2^{a}4^{b}$ mice. CH12 cells express both the I-A^{k} and I-E^{k} class II molecules [13,14]. Despite the tumorigenic properties of CH12, these cells possess at least some of the activation properties of resting B cells. Specific antigen and H-2-restricted interactions with Th cells lead to their differentiation into antibody-secreting cells [5,14]. CH12 cells also can be stimulated with lipopolysaccharide (LPS). LPS-mediated activation is not influenced by sIg-ligand interactions [14]. In some experiments, an in vitro propagated clone of CH12 was used; cloning was achieved by limiting dilution.

Hapten-Binding B Cells

Fluorescein (FL)-specific B cells were purified on FL-gelatin plates as described [15]. The majority of FL-binding B cells from spleens of unprimed mice have the activation properties of resting B cells [3,16]. Activated FL-binding B cells were prepared from spleens of mice primed seven days earlier with FL-ficoll [15]. Approximately 10% of these cells can be triggered to form antibody producing clones of 20–100 progeny cells [15].

Helper Assays and Enumeration of Antibody Secreting Cells

CH12 cells were cultured with irradiated (1500R) Th cells and SRBC or, as controls, mouse erythrocytes (MRBC) from 2^a4^b mice, as described [5,14]. FL-binding B cells were cultured with KLH-specific T cells, irradiated F1 spleen cells as APC, and various amounts of FL-KLH or KLH [3,15,16]. The number of antibody-secreting cells were counted by direct plaque assay on appropriate targets.

Measurement of T-Cell Activation

T-cell activation was measured by the release of T cell-derived lymphokines interleukin 2 (IL2) [11,17] and T cell replacing factor (TRF) [17] or by T-cell proliferation. T-cell proliferation was measured by ^{3}H-thymidine (^{3}H-TdR) uptake [9].

Monoclonal Antibodies

Monoclonal anti-I-A^k (10-6.3.2) [18] and anti-I-E^k (14-4-4S) [19] were obtained from the American Type Culture Collection (Rockville, MD). Culture supernatants were concentrated by $(NH_4)_2SO_4$ precipitation, the immunoglobulins were purified using protein A affinity chromatography, and protein content determined. Both monoclonal antibodies are IgG2a,κ. Both lysed CH12 at similar titers using a complement-dependent microcytotoxicity test (50% end point = 1/64 for 10-6.3.2, 1/128 for 14-4-4S). Cell sorter analysis has demonstrated previously that the I-A and I-E molecules are present at similar cell densities [14].

RESULTS

Requirement for sIg-Ligand Interactions in B-Cell Activation

KLH-specific Th cells, KLH, and the SRBC-specific B-cell lymphoma CH12 were cultured with SRBC or with MRBC as a control antigen. As shown in Table I, the presence of SRBC was required for Th cell-dependent activation of CH12; SRBC alone was insufficient. MRBC did not substitute for SRBC and, furthermore, did not interfere with the ability of KLH-specific Th cells to trigger CH12 in the presence of SRBC (experiment 2). Previous studies have shown that KLH is necessary for the activation of the Th cells used [5,11] (Fig. 1). As expected, LPS-mediated stimulation was achieved in the absence of SRBC [14].

Under the conditions employed, Th cell-dependent differentiation of CH12 requires two antigens, one for which the Th cells are specific (KLH) and a second, the antigen bound by CH12 (SRBC). This result is consistent with the interpretation that sIg-ligand interactions play an active role in B-cell activation. However, it is possible that SRBC enhances the ability of CH12 to present KLH to the Th cells. To

TABLE I. Requirement for T Cell Help and Specific Antigen in B-Cell Stimulation: No Need for Linked Interactions

Experiment	CH12 cultured with[a]	Erythrocyte antigen[b]	Activation of CH12: % of recovered cells ± SD[c]
1	—	MRBC	0.29 ± 0.03
	—	SRBC	0.67 ± 0.18
	T · KLH + KLH	MRBC	0.21 ± 0.03
	T · KLH + KLH	SRBC	3.78 ± 0.53
	LPS	—	7.26 ± 1.53
2	—	MRBC	0.99 ± 0.12
	—	SRBC	1.13 ± 0.41
	T · KLH + KLH	SRBC	5.55 ± 0.37
	T · KLH + KLH	SRBC + MRBC	6.06 ± 0.68

[a]All cultures contained 2×10^5 CH12 cells in a volume of 2 ml; 10^5 KLH-specific Th cells + 40 μg/ml KLH or 50 μg/ml LPS were added to appropriate cultures.
[b]2×10^6 MRBC from 2^a4^b mice or SRBC where designated.
[c]Expressed as anti-SRBC PFC measured after four days of culture.

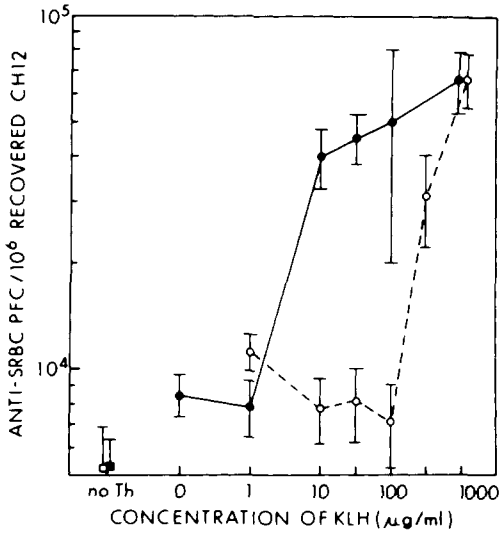

Fig. 1. Bypassing the requirements for sIg-ligand binding in B-cell activation. KLH-specific Th cells were cultured with increasing concentrations of KLH in the presence (●——●) or absence (○---○) of SRBC. PFC were measured on day three of culture. Vertical bars are standard deviations of quadruplicate determinations.

eliminate this possibility, this study determined whether the presence of SRBC influenced the level of either IL2 or TRF produced by activated Th cells in cultures of CH12. As shown in Table II, KLH-specific Th cells were effectively stimulated even when SRBC was not present. In other studies not shown, CH12 was found to present antigen as effectively as conventional (splenic) sources of APC. It is possible that the

TABLE II. Helper T-Cell Activation Does Not Require the Presence of SRBC in Cultures of CH12*

T · KLH added[a]	SRBC	Lymphokine in culture supernatants	
		IL2 (units/ml)[b]	TRF (pfc/10^6)[c]
−	+	0	19
+	−	21	141
+	+	18	138

*Cultures contained 2×10^5 CH12 cells and 2×10^6 SRBC where shown. Supernatants were harvested after 48 hr of culture.

[a] 10^6/culture (irradiated 1500R) + 40 μg/ml KLH.

[b] No IL2 was detected in supernatants from cultures of T · KLH and CH12 cells when KLH was omitted.

[c] Supernatants were added to a final concentration of 10% v/v. Addition of a supernatant containing IL2 but not TRF to these test supernatants (in order to ensure conditions such that TRF and not IL2 was limiting in culture) did not increase the number of SRBC-specific pfc. Cultures containing a standard TRF-positive supernatant (from concanavalin A-stimulated spleen cells) gave 311 pfc/10^6; cultures with SRBC, but no added supernatant, gave 26 pfc/10^6.

presence of SRBC, although not influencing TRF and IL2 production, improved the ability of Th cells to stimulate CH12. The authors believe that this is unlikely because, under the appropriate conditions, SRBC are not required for direct Th cell-CH12 interactions, leading to the induction of antibody secretion by CH12 (Fig. 1). It is thus concluded that the activation of Th cells requires the presence of the T-cell-specific antigen KLH, but not of the B-cell-specific antigen SRBC. Therefore, any differences in the activation of CH12 to antibody secretion between cultures containing SRBC and those lacking SRBC reflect the activation requirements of CH12 and not of the KLH-specific Th cells.

The requirement for sIg-ligand interactions in the Th cell-dependent activation of CH12 is not absolute. Conditions can be found under which the presence of SRBC is not necessary. As shown in Figure 1, the addition of supraoptimal concentrations of KLH (as defined by Th cell activation) resulted in the stimulation of CH12 in the absence of added SRBC. This effect is not due to mitogenicity of KLH, as these concentrations of KLH have no effect on CH12 in the absence of KLH-specific Th cells (not shown). It is possible that high concentrations of KLH cause nonspecific redistribution of sIg, resulting in an effect similar to that of specific sIg-ligand interactions [20]. Alternatively, high concentrations may allow the focusing of supraoptimal numbers of Th cells onto the CH12 surface, rendering the sIg molecule unnecessary in B-cell activation. This latter interpretation is supported by evidence obtained using alloreactive Th cells specific for Ia molecules on the CH12 surface. At high multiplicities of these Th cells, the requirement for sIg-ligand interactions can be bypassed [5].

The fact that the requirement for sIg-ligand interactions can be overcome is not unique to the CH12 lymphoma system. As shown in Figure 2, resting FL-binding B cells can be stimulated by KLH-specific Th cells when high concentrations of KLH are substituted for low concentrations of the specific conjugate FL-KLH (Fig. 2a,b). High concentrations of KLH are not required for the stimulation of activated B cells

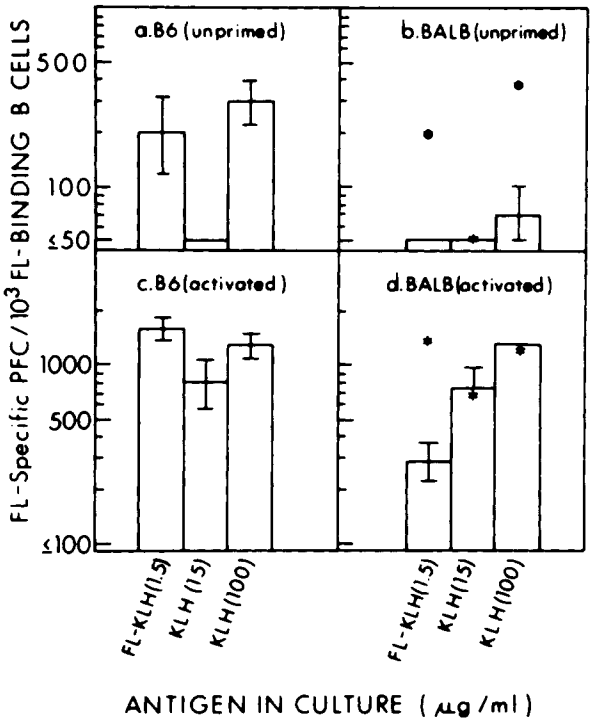

Fig. 2. Requirements for stimulating hapten-binding B cells differ depending on the activation state of the responding B cell. FL-binding B cells from unprimed or primed mice of the strains shown were cultured with the designated antigen, B6-restricted CB6F1 Th cells and CB6F1 APC. Results are FL-specific PFC per PFC per 10^3 input B cells on day four of culture; vertical lines represent SD of means of individual cultures. (*) Positive controls for BALB B cells using BALB restricted CB6F1 Th cells.

(Fig. 2c,d). Unlike resting cells, activated B cells are susceptible to the action of B-cell stimulating factors (BSF) released by T cells stimulated with low concentrations of KLH. Moreover, activated B cells are further stimulated, irrespective of the MHC type of the responding Th cell, as long as the Th cells are activated (2,3). However, resting B cells require MHC-restricted interactions with Th cells, regardless of whether the B cells are activated in the presence of hapten-carrier conjugates or high levels of carrier alone (compare Fig. 2a, b with c, d).

Role of MHC-Encoded Ia Molecules in B-Cell Activation

The requirement for MHC-restricted interactions between Th cells and resting B cells might reflect one of two mechanisms. First, resting B cells might be less sensitive to BSF than activated B cells and require high concentrations of BSF that are made possible by direct interactions with Th cells. Thus, the Ia molecule would serve a passive focusing role. Alternatively, Ia molecules might be receptors which, upon interaction with Th cells, are active in the transduction of an activation signal to the responding B cell as a direct result of binding with the Th cell receptor. In both cases, B-cell Ia molecules would serve as the restricting element for Th cell activation.

To distinguish whether Ia molecules have a passive or active role in B-cell stimulation, it was first determined whether Th cells directed against both I-region encoded molecules expressed on the CH12 membrane were equally effective in activating CH12 cells. Alloreactive Th cells specific for these molecules were generated and tested for their ability to stimulate CH12. Table III shows the surprising results of this experiment. Only Th cells directed against I-E^k (E^k_α), and not those directed against I-A^k, were efficient stimulators of CH12. In other experiments using established methods [10], it was confirmed that the alloreactive Th cells specific for either the I-A or the I-E molecules are capable of stimulating normal SRBC-specific B cells from 2^a4^b mice.

Although CH12 expresses similar levels of I-A^k and I-E^k molecules [14], it is possible that the I-A^k molecule has been altered in the tumor. Therefore, the failure to activate CH12 cells might reflect simply the failure to activate T-cell help. To test this, this study investigated whether CH12 cells could present antigen to I-A^k-restricted Th cells. As shown in Figure 3, KLH-specific T cells from B10.A(4R) mice were stimulated by KLH presented by cloned CH12 cells. These Th cells, however, did not stimulate CH12 to antibody secretion. In contrast, B10.A(3R) T cells, as well as 2^a4^b T cells, did stimulate CH12. Similar results have been obtained using SRBC-specific B10.A(4R) and B10.A(3R) Th cells. Even in the presence of normal splenic APC, functionally competent B10.A(4R) Th cells failed to stimulate CH12 [14].

The previous results could be explained by unexpected regulatory effects of cells contaminating the Th cell populations or by suppressive activity of the I-A^k-specific T cells. However, this seems unlikely. First, it should be noted that Th cells from H-2^a mice, which contain a mixture of I-A^k-restricted and I-E^k-restricted Th cells, as determined by antibody blocking studies and by the ability to clone functionally competent I-A^k-restricted Th cells from these populations [35], are competent to help CH12. Therefore, the failure of I-A^k-restricted Th cells to activate CH12 cannot be explained solely by a suppressive effect of these cells. Moreover, purified mono-

TABLE III. I Region-Encoded Molecules Are Involved in Transmembrane Signalling of T-Cell Help to the CH12 Lymphoma

	Specificity of alloreactive helper T-cell lines[a]				SRBC	Activation of CH12:
	K	$A_\beta A_\alpha E_\beta$	E_α	D	in culture	% of recovered cells[b]
i)	k	k	k	—	−	6.3
					+	17.6
ii)	—	—	k	d	−	5.6
					+	17.8
iii)	k	k	—	d	−	2.7
					+	2.7
		none			+	2.6

[a]Th cells were derived from primary and secondary MLCs using the following combinations: i) (CB6F1 anti-2^a4^b) anti-C3H; ii) (CB6F1 anti-2^a4^b) anti-B10.A(3R); iii) B10.A(5R) anti-B10.A(2R).

[b]Expressed as percent of cells secreting antibody; anti-SRBC PFC were measured on day three of culture.

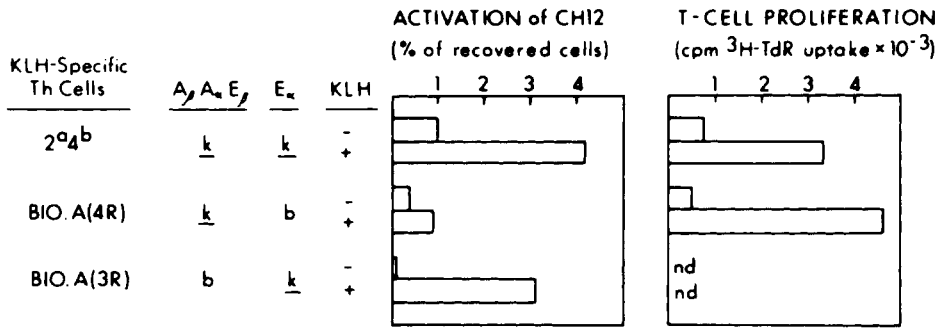

Fig. 3. Activation of Th cells by antigen presented by CH12 does not always result in the activation of CH12. KLH-specific Th cells (10^4) of the designated strains were cultured with CH12. Activation of CH12 was measured by the production of anti-SRBC PFC; Th cell activation was measured by ^{3}H-TdR incorporation. Where T-cell proliferation was measured, a cloned in vitro line of CH12 was used; CH12 was treated with mitomycin C to inhibit its proliferation.

clonal antibody to the I-E^k molecule, but not the I-A^k molecule, stimulates CH12 (Fig. 4). Similar results have been obtained using three different anti-I-A^k and eight unique anti-I-E^k monoclonal antibodies [LoCascio et al, in preparation]. This result raises intriguing questions about the nature of Th cell receptor-Ia interactions leading to B-cell excitation.

DISCUSSION

These data provide evidence that both sIg and Ia molecules on the B-cell membrane contribute to the activation of (at least) resting B cells. A schematic representation of our model of B-cell activation is presented in Figure 5. Interaction of the appropriate ligands with both sIg and Ia molecules results in an initial excitation signal(s) to the resting B cell. This induces the resting B cell to leave the G_0 cell cycle state and enter G_1. The ultimate result of these interactions is the expression of (preselected?) gene programs in "excited" B cells. At least some of these new gene products must include receptors for various BSFs, including factors that promote B-cell proliferation and differentiation [21], as activated as opposed to resting B cells no longer require specific antigen and MHC-restricted interactions for their further stimulation (Fig. 2). Recent evidence shows that a sequential expression of BSF receptors is likely to occur and is influenced, at least in part, by whether these receptors are occupied by homologous factors [22,23]. Whether sIg-antigen binding and restricted interactions between Th cells and B-cell Ia influence the quality of the response once B cells have been stimulated from a resting state is unknown, but clearly these interactions are not needed for clonal expansion and antibody secretion to occur [1–3,16] (Fig. 2).

Although antigen bridging by hapten-carrier conjugates leads to both sIg-hapten binding and Th cell-Ia interactions, these two components of B-cell activation need not be given in a linked form. The independent and separate binding of antigen to sIg and T-cell help to Ia molecules clearly initiate B-cell activation (Table I) [4,5]. From our studies it is unclear whether Th cell-Ia interactions and sIg-ligand binding each

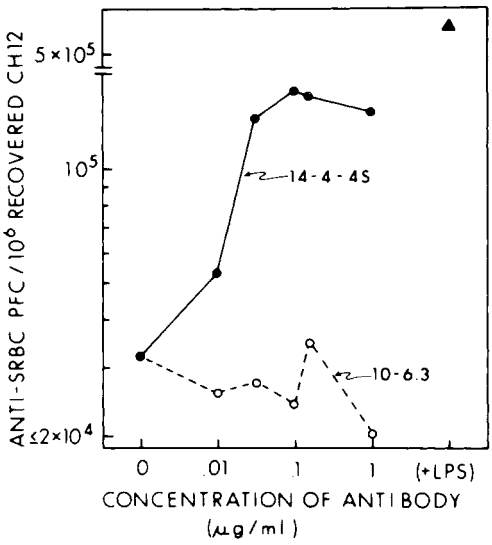

Fig. 4. Monoclonal antibody to I-E^k but not I-A^k stimulates CH12. All cultures included SRBC. Experiments not shown demonstrated that under the conditions used the presence of SRBC was essential for anti-I-E-mediated stimulation. Results are using anti I-E^k antibody 14-4-4S (●), or anti I-A^K antibody 10-6.3 (○). Both antibodies were purified by protein A affinity chromatography before use. (▲) represents response to 50 μg per ml LPS.

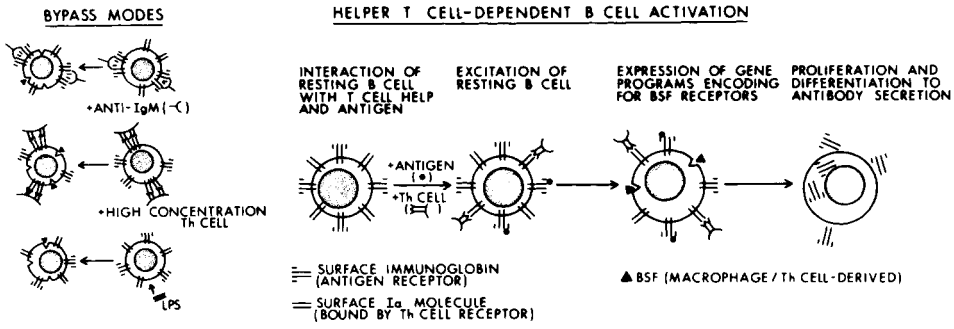

Fig. 5. Schematic representation of the role of sIg and Ia molecules in B cell excitation. See Discussion for explanation.

give an independent "signal" to the resting B cell. It is well documented that most so-called T-dependent antigens, when bound by sIg, do not alter the cell cycle state or activation properties of a resting B cell [3,16,24]. These results, which show that SRBC has little or no effect on CH12, unless T-cell help is given concommitantly, are consistent with this. Moreover, under conditions in which "low-multiplicities" of Th cells interact with B cells [5], Th cell-Ia interactions also do not lead to B-cell activation; unless the sIg molecule is also occupied (Table I). It is possible that entry into the cell cycle is an irreversible event that occurs only when sufficient numbers of Ia and/or sIg molecules are occupied. If true, it would follow that under unusual conditions in which high numbers of sIg or Ia molecules are bound (cross-linked?)

the requirement for ligand interactions with the other molecule would be bypassed. This seems to be the case.

The binding of antigen to sIg, T-cell help to membrane Ia, or both, need not be essential for the activation of resting B cells. Anti-immunoglobulin reagents are capable of stimulating resting B cells to proliferate, bypassing the requirements for interactions with Ia molecules [25]. However, BSF is still required for the further proliferation and differentiation of anti-Ig stimulated B cells [25,26]. As demonstrated with both normal B cells (Fig. 2; ref.3,20) and CH12 (Fig. 1) [5], sufficient levels of Ia-specific T-cell help are capable of bypassing any requirements for specific sIg-ligand interactions. BSF is likely to be required under these conditions, at least in responses of normal B cells. The ability of LPS to stimulate resting B cells [2,27] is evidence that both sIg and Ia can be bypassed. As is the case with other modes of B-cell activation, certain BSFs are still required for the differentiation of LPS-stimulated B cells [28].

The study of the CH12 lymphoma has provided some interesting insights into the role of surface Ia in B-cell activation. First, helper T cells specific for the I-E^k but not I-A^k molecules are capable of triggering CH12, demonstrating that only one of two expressed Ia molecules is functional in transducing a signal upon interaction with Th cells. Because the I-E but not the I-A molecules are active, irrespective of T-cell specificity (SRBC-specific T cells [14] as well as allospecific and KLH-specific Th cells show this effect), it is unlikely that the apparent limitation in specificity is due to Ir gene control of T-cell function. Rather, these results are interpreted as demonstrating a specific inability of the I-A molecule on CH12 to serve this receptor function. The possibility that the I-A molecule on CH12 has a function not measured by our assays cannot be ruled out, but this function is clearly not one of induction of antibody secretion [this report] nor of proliferation [LoCascio et al, in preparation]. It is unknown whether only one of the Ia molecules on *individual* normal B cells is a functional receptor, as with CH12. If so, this might provide a molecular basis of the phenomenon termed "adaptive differentiation" of B cells [29,30]. Clearly, however, many B cells can be triggered by I-A-specific T-cell help. It is possible that all normal B cells can be stimulated via both Ia molecules (in those strains expressing I-A and I-E) and that the I-A molecule on CH12 is in some way defective.

A monoclonal anti-I-E^k antibody was capable of stimulating antibody secretion from CH12. Similar results using anti-DR antibodies and normal human B cells were reported first by Palacios et al [31]. The ability of monoclonal antibodies to substitute for T-cell help in the activation of CH12 implies that the initial requisite interaction between Th cells and Ia molecules is an "inert" one between receptor and ligand, and not necessarily a metabolically dependent process on the part of the T cell. Although it is not clear that one can equate T-cell receptor binding to Ia molecules with antibody to Ia, this is a testable hypothesis. If true, it suggests that receptor-Ia interactions lead to the activation of B cells as a result of an internal change in the B cell due to the receptor nature of the Ia molecule, rather than as a secondary function of any T cell-derived activation signal. Whether CH12 requires any of the subsequent signals from activated T cells (BSF) required by normal B cells following the initial activation process is unknown.

The observation that the I-A^k product on the CH12 surface will serve as a restriction molecule for KLH-specific Th cells, even though this interaction will not lead to B-cell activation, demonstrates that these two functions of the Ia molecule are

separable and distinct. Although Ir gene control of T-cell function is largely thought to reflect structural or conformational differences contributed by the more polymorphic β chain of Ia molecules [32,33], matching for the E^k_α chain is sufficient to result in activation of CH12 (14). Moreover, KLH-specific B10.A(3R) T cells activate CH12 (Fig. 2), while KLH-specific CB6F1 T cells do not (5). Both come from strains which express an I-E molecule composed of an E^b_β gene product, whereas the E^k_α expressed on B10.A(3R) varies slightly from the E^d_α of CB6F1 [33]. It is noteworthy that CB6F1 T cells are stimulated in cultures containing CH12 to produce T-cell-derived lymphokines [5]. Evidently, differences in the E_α chain between the two strains are sufficient to explain the stimulatory differences observed. This study cannot, or course, rule out a role of conformation in either function of Ia molecules, namely T-cell activation and B-cell stimulation. A conformational determinant, Ia 22, shared by I-E molecules composed of $E^k_\beta E^k_\alpha$ and $E^b_\beta E^k_\alpha$, expressed in 2^a4^b and B10.A(3R) mice, respectively, has been described using monoclonal antibodies [34]. Further experiments to examine these questions are in progress.

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