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Immunology

Key antigenic determinants in regulation of the immune response

(suppressor cells/T-cell interactions/T-cell triggering)

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ABSTRACT The immune response to β-galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23) is characterized by a wave of early help followed by a wave of suppression to a subsequent *in vitro* challenge with galactosidase-fluorescein. A cyanogen bromide peptide of β-galactosidase, CB2, mimics the suppression seen with the enzyme. It is time dependent, carrier specific, and anti-theta sensitive; however, this supression is not preceded by a wave of help. It is possible that CB2 cannot stimulate helpers, and is only able to activate suppressor cells. These data indicate that one small region of an antigen, capable of activating suppressors, can nullify the positive effect induced in helper T cells reactive with other epitopes on β-galactosidase. Key determinants on macromolecules may in this way be influential in regulating the immune response to the entire antigen molecule.

Some experimental evidence exists suggesting that subregions of a molecule may regulate the immune response to the entire antigen. Hill and Sercarz (1) have suggested that a single epitope differing in two closely related lysozymes can determine responsiveness in H-2^b mice. Swanborg and coworkers (2, 3) and others (4) have demonstrated that the antigen, myelin basic protein, which can induce the disease experimental allergic encephalomyelitis, can be cleaved into distinct regions, one of which can cause the encephalitis and another of which can specifically turn on suppressor cells which prevent the disease upon subsequent challenge with myelin basic protein.

These data strongly suggest that certain epitopes may be able to activate only one of the major subfractions of T cells, helpers or suppressors. In this report, we describe a system in which a cyanogen bromide cleavage peptide of β-galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23), CB2, induces a wave of suppression specific for haptens coupled to the β-galactosidase. Unlike the sequential generation of suppressors and helpers shown for the β-galactosidase molecule as a whole (5, 6), CB2 does not cause an early or a later wave of help. It is probable that CB2 possesses influential determinants that regulate the course of the response to the entire β-galactosidase molecule.

MATERIALS AND METHODS

Animals. Eight- to 12-week-old CBA/J mice were obtained from the Jackson Laboratories, Bar Harbor, ME.

Antigens. β-Galactosidase was prepared by the method of Fowler (7). Cyanogen bromide peptides of the enzyme were the generous gifts of K. Langley, A. V. Fowler, and I. Zabin, Department of Biological Chemistry, University of California, Los Angeles. Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem. Fluorescein (FL) isothiocyanate was obtained from Baltimore Biological Laboratories. β-Galactosidase and KLH were fluoresceinated by the method of Goldman (8). Briefly, 10 mg of antigen in 1 ml of carbonate buffer (pH 9.5) was mixed with 300 μg of solid fluorescein isothiocyanate and the reaction continued for 6 hr at room temperature. The reaction was stopped by passage over Sephadex G-25 to separate the conjugate from the uncoupled fluorescein. FL_{21}-galactosidase and FL_{48}-KLH were the preparations used throughout these experiments.

Immunizations. One hundred micrograms of β-galactosidase, 100 μg of KLH, or 5 μg of cyanogen bromide peptides were emulsified in complete Freund's adjuvant (Gibco) and injected intraperitoneally at various times prior to spleen culture.

Cell Culture and Plaque Assay. Spleens were removed and cells cultured by the method of Mishell and Dutton (9). The antigen dose *in vitro* was routinely 1 μg/ml. Cultures were assayed for direct plaques after 4 days by the hemolytic plaque assay of Cunningham and Szenberg (10). Dashes in the tables refer to less than 3–10 plaque-forming cells (PFC) per culture. Fluorescein-substituted goat erythrocytes were prepared by the method of Wolf *et al.* (11). Fluorescein isothiocyanate (5 mg) in 1 ml of carbonate-buffered saline (pH 9.5) reacted for 1 hr at 0° with 0.25 ml of packed goat erythrocytes followed by six washes in phosphate-buffered saline (pH 7.2). Fresh guinea pig serum absorbed with goat erythrocytes was used as a source of complement at a final dilution of 1:32.

Anti-Theta Treatment. AKR/J mice were immunized with CBA/J thymocytes weekly in increasing doses of cells from 1×10^7 the first week to 4×10^7 the fourth and subsequent weeks. Serial bleedings were taken and titrated by the procedure described by Raff (12), except that trypan blue exclusion was used as the criterion for viability. A dilution of 1:20 of anti-theta serum gave a plateau value of 35% of splenic lymphocytes. Commercial lyophilized guinea pig serum (Baltimore Biological Laboratories) absorbed with agarose (80 mg/ml) was used as a source of complement at a final dilution of 1:4, sequential to antiserum treatment.

RESULTS

Successive Waves of Help and Suppression to β-Galactosidase. *In vivo* priming with β-galactosidase initiates T-cell activity which can be measured at intervals by spleen cell culture with β-galactosidase attached to haptens such as FL. The first demonstrable activity ("early help") is a short-lived helper effect peaking at 3 days. Early help to β-galactosidase is followed by a wave of suppression that is maximal on day 7 after priming. This phase is also temporary, and by 2 weeks the helper effect again predominates (5). The sequence and duration of these activities seems to vary as a function of the hapten and its density on the carrier protein (6, 13, 14).

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Abbreviations: CB2, second cyanogen bromide cleavage peptide of β-galactosidase; KLH, keyhole limpet hemocyanin; FL, fluorescein; PFC, plaque-forming cells.

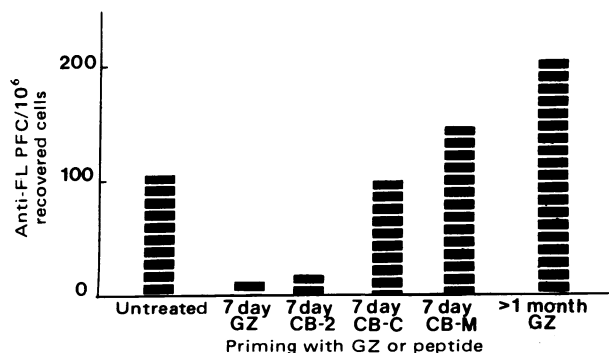

FIG. 1. CBA/J mice (6–8 weeks old) were injected with 100 μg of β-galactosidase (GZ) in complete Freund's adjuvant or with 5 μg of cyanogen bromide (CB) peptides in the adjuvant. Seven days, or at various times after immunization, spleens were put into Mishell–Dutton culture with 1 μg of galactosidase-FL per ml. Four days later, direct anti-FL PFC were enumerated. The level of CB2 suppression approximated β-galactosidase suppression in each of eight experiments.

CB2 Suppresses Response to Galactosidase-FL. β-Galactosidase naturally occurs as a tetramer of four identical subunits of 116,250 molecular weight. CB2 is the second cyanogen bromide peptide of β-galactosidase from the NH_2-terminal end of the monomer, comprising amino acid residues 3–92. Two other peptides were used, one from the middle of the monomer (termed CB-M, molecular weight $\simeq$ 7500) and the COOH-terminal peptide (termed CB-C, molecular weight $\simeq$ 3600) (A. Fowler and I. Zabin, unpublished). In order to see whether subregions of β-galactosidase could modulate the *in vitro* anti-hapten response to galactosidase-FL, these three cyanogen bromide peptides were injected into different sets of CBA/J mice 7 days before their spleens were removed for culture *in vitro*. As Fig. 1 shows, two of the peptides tested had little effect on the subsequent galactosidase-FL response. However, mice primed with CB2 7 days earlier had a greatly reduced ability to respond to galactosidase-FL *in vitro*. The level of CB2 suppression approximated β-galactosidase suppresion in each of eight experiments.

One hundred micrograms of β-galactosidase is the optimal dose for inducing the suppression, both in an adoptive transfer and an *in vitro* culture system (6, 15). Doses of CB2 in the same molar dose range were examined for their ability to induce suppression. Each of the three doses tested (Table 1) induced suppression, although the 5-μg dose seemed to be most inhibitory. Therefore, 5 μg of CB2 was chosen as the dose for induction of suppression in later experiments.

Lack of Early Induction of Help by CB2. We then investigated whether CB2 induced early help as had been shown for β-galactosidase. When mice were primed with CB2, they failed to give the early help response. Table 2 shows that 3 days after priming with CB2, there was no increase in the response to galactosidase-FL compared to that in normal spleens, but by day 7, there was an absence of responsiveness.

Table 1. Dose titration of CB2

CB2, μg	Anti-FL PFC/ 10^6 recovered cells	Average % suppression ± SE
1	29 ± 16	73 ± 15
5	9 ± 5	93 ± 4
10	65 ± 36	66 ± 35

Different sets of CBA/J mice were injected with 1, 5, or 10 μg of CB2 in complete Freund's adjuvant for priming. After 7 days, their spleens were removed and put into Mishell–Dutton culture with 1 μg of galactosidase-FL per ml. Four days later, direct anti-FL PFC were enumerated. Results are expressed as the percent of response in unprimed spleen cells. The data are averaged from two separate experiments for the 1- and 10-μg doses, and from four separate experiments for the 5-μg dose.

Table 2. CB2 does not prime for early help

Priming *in vivo*	Anti-FL PFC/10^7 cultured cells Exp. 1	Exp. 2
None	97	80
CB2, 3 days	80	105
CB2, 7 days	0	0
β-Galactosidase, 3 days	245	200
β-Galactosidase, 7 days	25	20
β-Galactosidase, ≥1 month	311	140

CBA/J mice (6–8 weeks old) were injected with 100 μg of β-galactosidase in complete Freund's adjuvant or 5 μg of CB2 in the adjuvant. At times noted, spleens were removed and put into Mishell–Dutton culture with 1 μg of galactosidase-FL per ml. Four days later, direct anti-FL PFC were enumerated.

T-Cell Dependence of CB2 Suppression. To determine whether the CB2 effect was truly suppressive and related to T cells, we mixed spleen cells from CB2-primed mice with normal spleen cells, both before and after treatment with anti-theta serum and complement. It is clear from the single experiment in Table 3 that the spleen cells from the CB2-primed mice were capable of turning off the response by normal cells. Anti-theta treatment alleviated the suppression and restored the response. In other unpublished experiments, using anti-Ly sera (16, 17), the suppressive cells were also shown to belong to a T-cell subpopulation.

Specificity of Suppression. The specificity of the suppression was tested (Table 4). Different mice were injected intraperitoneally with CB2, β-galactosidase, or KLH in complete Freund's adjuvant 7 days before their spleens were cultured. The *in vitro* responses to galactosidase-FL, KLH-FL, and sheep erythrocytes were examined. The anti-fluorescein response to KLH-FL, and the anti-sheep erythrocyte response were unaffected by CB2 priming, while the response to galactosidase-FL was severely depressed.

Moreover, even if soluble β-galactosidase was added to cultures of CB2-primed cells challenged with KLH-FL, a normal anti-fluorescein response ensued. This suggests that no soluble mediators are released that might nonspecifically suppress unrelated responses.

DISCUSSION

Transmission of Suppressive Effect from One Determinant throughout the Molecule. We have shown that CB2, a peptide of β-galactosidase comprising 9% of its sequence, can be responsible for eliciting suppressor cells which will affect the response to any determinant attached to the entire β-galactosidase molecule. This suppression is anti-theta sensitive, antigen

Table 3. Dominant suppression by CB2 is anti-theta sensitive

Normal cells	CB2-primed cells	Anti-FL PFC/ 10^7 cultured cells
10^7	—	203
—	10^7	13
5×10^6	5×10^6	6
5×10^6	5×10^6*	183

See legend of Table 2 for experimental protocol. Direct anti-FL PFC were enumerated 4 days after the onset of culture.
* Treated with anti-theta serum.

Table 4. Specificity of suppression with CB2

Priming *in vivo*	Anti-FL or anti-SRC PFC/10^6, from cultures immunized with: GZ-FL	KLH-FL	KLH + GZ-FL	GZ + KLH-FL	SRC
None	203	226	203	196	1008
β-Galactosidase, 7 days	15	193	—	224	1121
β-Galactosidase, >1 month	470	179	—	—	889
KLH, 7 days	464	58	171	—	1264
KLH, >1 month	290	446	—	—	1370
CB2, 7 days	13	270	—	275	1301

CBA/J mice (6–8 weeks old) were injected, in complete Freund's adjuvant, with 100 μg of β-galactosidase, 100 μg of KLH, or 5 μg of CB2. At various times after immunization, spleens were put into Mishell–Dutton culture with 1 μg of soluble carriers and/or hapten-carrier conjugates per ml as noted above or with 5×10^6 sheep erythrocytes (SRC). Four days later, direct anti-FL or anti-SRC PFC were enumerated. The results expressed are representative of those obtained in three separate experiments. GZ-FL, galactosidase-FL.

specific, and time dependent. Two other small cyanogen bromide peptides did not induce suppression or help. CB2 can therefore be considered to possess at least one important suppressive epitope whose effect is transmitted throughout the antigenic molecule, nullifying the expression of all β-galactosidase helper cells.

Key Suppressive Determinants May Be Implicated in Genetic Control of Immune Responses. A similar situation appears to exist in several systems of genetic control of immune responsiveness, in which carrier-induced suppression seems to be exaggerated so that the suppression is quite prolonged. Hill and Sercarz (1) have shown that the H-2^b mouse strain is nonresponsive to chicken lysozyme but highly responsive to Japanese quail lysozyme. These two lysozymes differ by six amino acid residues, all of which cluster on one side of the molecule. Here a signal generated by a key suppressive determinant on chicken lysozyme, but not present on quail lysozyme, affects T-helpers directed against other parts of chicken lysozyme. A somewhat similar case has been reported (18, 19) in which a polypeptide of glutamic acid and alanine, which is immunogenic in H-2^s mice, is converted into a nonimmunogenic peptide for this strain by the addition of 4–10% tyrosyl residues. In our system, we assume that a minority of putative "suppressor determinants" on β-galactosidase offset the majority of β-galactosidase helper determinants.

Other Systems Exhibiting Suppressive Antigenic Determinants. The regulatory role of key determinants has also been reported in other systems in which suppressor cells play a role, both in generation of anti-hapten responses (20) and where certain cyanogen bromide peptides from myelin basic protein affect the autoimmune process differentially, some causing suppression of disease and others mimicking the entire protein in causing encephalomyelitis (2–4).

Interaction between Helper and Suppressor T Cells after CB2 Priming. These data lend strong inferential support to a model involving T–T cell interaction. Since the CB2-primed suppressor cells seem to inactivate the potential responsiveness of T helper cells for the rest of the β-galactosidase molecule, a T–T interaction is suggested, presumably through the Ly subclasses of T cells (16, 17).

We know that CB2 can trigger suppressor cells, and furthermore that it does not generate early help. It is probable that CB2 is unable to trigger Ly 1 cells, although it is possible that helper T cells are raised to some extent by CB2, but the suppressors mask their expression, as was noted for the polypeptide Glu, Ala, Tyr (18, 19). There is direct testing of these hypotheses underway.

Preferential Interaction by Antigens with a Single T-Cell Subclass. The mechanism by which an antigen can preferentially interact with one subpopulation of cells is unclear. It may be that subclasses of T cells have different libraries of receptors, i.e., Ly 1 cells have receptors with specificities for antigenic determinants different from those present on the Ly 2,3 cells. Thus, by merely seeking its receptor, the antigen selects for the function the T cell will express. This would be unlikely if both Ly 1 and Ly 2,3 cells were derived from the same Ly 1,2,3 precursor, but there could very well be two subsets of Ly 1,2,3 cells, one destined to become suppressive and the other helpful.

An alternative hypothesis would be that the antigen selects a population of T cells by differential association with cell surface molecules. Since T-cell specificity seems to involve recognition of an antigenic determinant in the context of a product of the major histocompatibility complex (21, 22), we may make the assumption that the presentation of selected portions of a complex antigen molecule may only occur with particular major histocompatibility complex molecules—K, D, or I. On the presenting cell surface, certain β-galactosidase determinants, for unknown reasons of chemical affinity, would associate with I-region products whereas others, such as CB2, would preferentially associate with K or D. This is schematically represented in Fig. 2. In this way, the decision about which of the T cells will be triggered by an antigenic determinant may be made at the surface membrane of a third-party cell. Diversity within the class of antigen-presenting cells may provide a further refinement in the selection and activation of T-cell populations.

Key Determinants Influence the Balance between Help and Suppression. Regulatory mechanisms seem to involve a delicate balance between positive and negative signals. The dominance of help or suppression at the level of the organism is usually considered a result of the numbers of each of these opposing cell types triggered by the antigen. It seems, from our

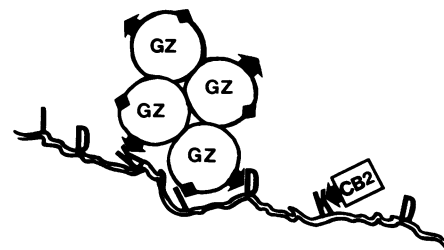

FIG. 2. Hypothetical representation of differential recognition of key determinants at the surface of a third-party cell, through interaction with gene products of the major histocompatibility complex. On the left, the β-galactosidase (GZ) tetramer interacts with K, D, and I; on the right, CB2 cannot interact with I.

work and that of others, that certain characteristic structures on an antigen may predominate in influencing the response to other epitopes on the molecule. In the most exaggerated cases, this might lead to a prolonged (and heritable) inability to mount a response against an antigen bearing such an epitope.

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