IMMUNOLOGICAL UNRESPONSIVENESS TO NATIVE DEXTRAN B512 IN YOUNG ANIMALS OF DEXTRAN HIGH RESPONDER STRAINS IS DUE TO LACK OF Ig RECEPTOR EXPRESSION

Evidence for a Nonrandom Expression of V-Genes*

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Two different types of genetically determined unresponsiveness to the α-1-6 epitope of the thymus independent (TI)[1] antigen dextran B512 have been previously described.

One type is illustrated by the congenic strains on A background, which cannot produce antibodies directed against dextran B512 (1). This unresponsiveness was found to be due to a failure of these strains to express Ig receptor against α-1-6 of dextran (1) and responsiveness was found to be linked to two particular allotypes of the heavy chain of immunoglobulins (unpublished observations), which were different from those associated with responsiveness to other TI antigens, such as the α-1-3 allotype of dextran B1351 and phosphorylcholine (2, 3).

Another example is found in the strain CBA/N, which is a nonresponder to several TI antigens, such as Ficoll and dextran and any hapten coupled to these carriers (1, 4, 5). The gene responsible for this unresponsive state has been localized to the X chromosome (5), and this strain lacks a subpopulation of B cells that can respond to the PBA properties on native dextran, B512 or else the relevant polyclonal B-cell-activating (PBA) receptors are absent (1).

In this paper we report a different mechanism of unresponsiveness to α-1-6 of dextran B512, which is restricted to young mice of high responder strains to the α-1-6 epitope. The immunological competence of these young animals was not generally affected, since they could mount a normal response against all thymus-dependent antigens like SRC and also to all TI antigens tested, such as the fluorescein isothiocyanate (FITC) epitope coupled to dextran.

In the present paper we have analyzed in detail the mechanism responsible for unresponsiveness of young mice to the α-1-6 epitope of dextran B512 and found that regulatory mechanisms, such as deficient T-cell help or the existence of suppressor cells could not account for unresponsiveness. Cells from nonresponder young mice were fully competent to respond to the PBA property of

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[1] *Abbreviations used in this paper:* BSS, ice-cold balanced salt solution; Dx, dextran; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; PFC, plaque-forming cell; SRC, sheep erythrocyte; TI, thymus independent.

dextran. The failure to produce antibodies against the α-1-6 epitope of dextran B512, was found to be caused by a temporary failure to express the V gene coding for this specificity.

Materials and Methods

Mice of the following strains were used in the present study: CBA, C57BL, and B10.5M and certain F_1 hybrids between these strains.

Antigens and Polyclonal Activators. Native dextran from *L. mesenteroides* B512 (average mol wt 5–40 × 10^6 daltons), was obtained from ICN Pharmaceuticals Inc., Life Sciences Group, Cleveland, Ohio. Other dextran preparations were obtained from Pharmacia, Uppsala, Sweden.

Native FITC-dextran was synthesized from native dextran B512 by reacting it with FITC, and was provided by Dr. van de Belder, Pharmacia, Uppsala. The final conjugation ratio was one molecule of FITC for every 200 glucose residues.

Lipopolysaccharide from *Escherichia coli* O55:B5 was prepared by phenol water extraction by Professor T. Holme (Department of Bacteriology, Karolinska Institutet).

Preparation of Lymphocytes. Spleens were removed and teased with forceps in ice-cold balanced salt solution (BSS). After brief sedimentation, the cells in the supernate were washed three times in 50 ml of cold BSS and subsequently suspended in culture medium to the desired cell concentration. Cellular viability was determined in a hemocytometer after staining the damaged cells with 0.02% trypan blue.

Assay of Antibody Synthesis. Anti-α-1-6 plaque-forming cells (PFC) were detected by a direct PFC assay with sheep erythrocytes (SRC) sensitized with stearoyl dextran B512 with a mol wt of 70,000 daltons, as described before by Howard et al. (6).

Anti-FITC PFC. The coupling of FITC to SRC has been described before (7). For detection of high, medium, and low affinity PFC the following concentrations of FITC in carbonate bicarbonate buffer pH 9.2 were used: 0.05 (or 0.1), 0.5, and 5 mg/ml.

Medium. The medium used in most of the experiments was Eagle's minimum essential medium in Earle's solution, supplemented with glutamine, nonessential amino acids, and pyruvate and containing 100 international units of penicillin and 100 μg of streptomycin/ml, as described by Mishell and Dutton (8). The medium was further buffered by 10 mM of Hepes and the pH adjusted to 7.2. All these reagents were obtained from Flow Laboratories, Irvine, Scotland. Most experiments were carried out in serum-free medium (9), except where specifically indicated in the figure legends.

Culture Conditions. For the in vitro experiments we used two different culture methods: high density cultures, which were carried out in serum-free medium as described before (9) with a cell density of 10^7 cells per ml. The cultures were set up in 1 ml vol in 3-cm plastic Petri dishes and were activated with lipopolysaccharide (LPS) (100 μg/ml culture) or native dextran (5–10 mg/culture) and the response tested at day 2 against SRC as well as SRC coupled with stearoyl dextran or FITC. Low cell density cultures contained 3 × 10^5 cells/ml in 2 ml medium (10). The cultures were supplemented with 5% human A serum and 2 mercaptoethanol at a final concentration of 5 × 10^{-5} M. The mitogens were used at the same concentration as above and the response was tested 4 days later.

Cell Transfer. Cells were washed in BSS and inoculated directly into irradiated recipients (600 rads). Mice were reconstituted between 2 and 5 h after irradiation and each animal received between 25 and 40 × 10^6 spleen cells i.v. with or without an immunogenic dose of 5 μg per animal of native dextran at the same time as reconstitution. The specific anti-α-1-6 response was tested 5 days after immunization.

Results

Nonresponder Young Animals to the α-1-6 Epitope of Dextran Respond Normally to Haptenated Dextran. It has been found that the response to the α-1-6 epitope of dextran in high responder strains matures very late in comparison with the response to all other antigens studied (11). At day 55 of age the number of PFC per spleen detected after immunization against dextran was

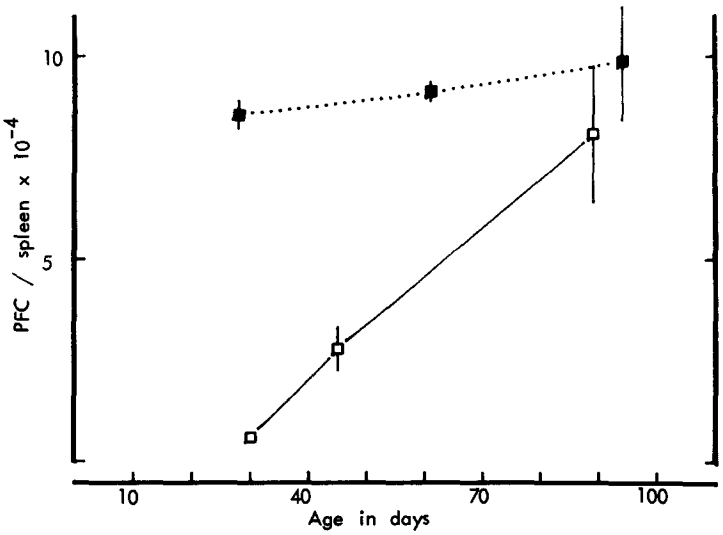

FIG. 1. The immune response to the α-1-6 epitope of dextran (Dx) (□——□) or to the hapten FITC (■· · · ·■) in C57BL mice of different ages. The mice were immunized i.v. with body weight adjusted doses of native dextran (corresponding to 10 μg/20 g) or FITC-dextran (100 μg/20 g). 5 (with dextran) or 4 (with FITC-dextran) days later the immune response was determined against SRC coated with 40 μg/10 ml of stearoyl dextran or with 0.5 mg/ml of FITC.

approximately 10% of the number obtained with animals 3 mo of age. Animals below 1 mo of age were complete nonresponders to the α-1-6 epitope.

Mice of the high responder strain C57BL varying in age from 29 to 94 days, were immunized i.v. with native dextran or FITC-dextran giving a body weight adjusted dose corresponding to 10 μg or 100 μg/20 g body weight, respectively. The immune response was tested at day 4 for medium affinity anti-FITC PFC and at day 5 for anti-α-1-6 PFC.

The results in Fig. 1 show the response against both epitopes. There is no significant difference between the anti-FITC response in FITC-dextran immunized mice irrespective of age, whereas there was a sharp increase of the anti-α-1-6 PFC with increasing age.

Cells from Dextran Unresponsive Young Mice Possess PBA Receptors for Dextran. Dextran was found to be an excellent carrier for the hapten FITC in young dextran nonresponder animals, and therefore it is likely that the B cells from young animals possess PBA receptors for dextran, since these are necessary for a TI response (12). However, the possibility that dextran unresponsiveness could be due to the lack of PBA receptor was critically investigated by stimulating cells from young animals with polyclonal doses of native dextran. Spleen cells from B10.5M and C57BL mice were cultured in vitro with both high and low cell density cultures. As can be seen from Table I and Fig. 2, spleen cells from all the animals tested – irrespective of age – responded equally to native dextran as a PBA, and were activated to polyclonal antibody synthesis against unrelated antigens.

Nonresponsiveness in Young Animals is Not Due to Suppressor Effects. 25 $\times$ 10^6 spleen cells from 1 or 6 mo old (A $\times$ CBA)F_1 mice were transferred into

TABLE I

*Polyclonal Induction of Antibody Synthesis in Vitro by Native Dextran and LPS with Cells From B10.5M Mice of Different Ages**

Age	PBA‡	PFC/10^6 recovered cells against	
		SRC	FITC§
10–14 days	–	4	76
	Dextran	64	610
	LPS	82	630
1 mo	–	7	117
	Dextran	40	440
	LPS	29	313
3 mo	–	4	74
	Dextran	15	219
	LPS	16	269

* Serum-free high density cultures were used as described in Materials and Methods. The response was tested at day 2.

‡ 1 mg/ml of native dextran and 100 μg/ml of LPS were added to the cultures.

§ A high (5 mg/ml) epitope density of FITC on SRC was used.

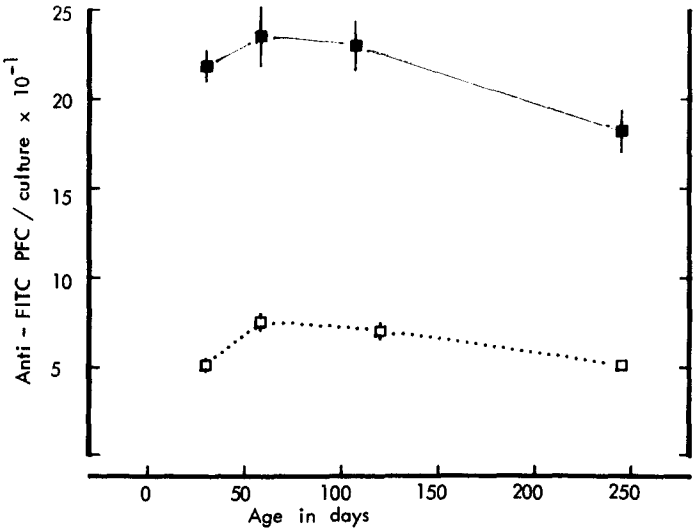

FIG. 2. The ability of native dextran to polyclonally activate antibody synthesis in spleen cells from C57BL mice of different ages. The cultures were given 1 mg native dextran/ml (■——■) or were left untreated (□· · · · □). Low cell density culture systems were employed as described in Materials and Methods. The response was tested at day 4 with heavily substituted FITC-SRC as targets.

lethally irradiated 3 mo old syngeneic recipients, which were immediately immunized with dextran. Control animals of the same ages were directly immunized and both groups were tested 5 days later for the PFC response against α-1-6. It was found that young animals, as well as cells from young animals transferred to old animals, were unable to respond to the α-1-6 epitope,

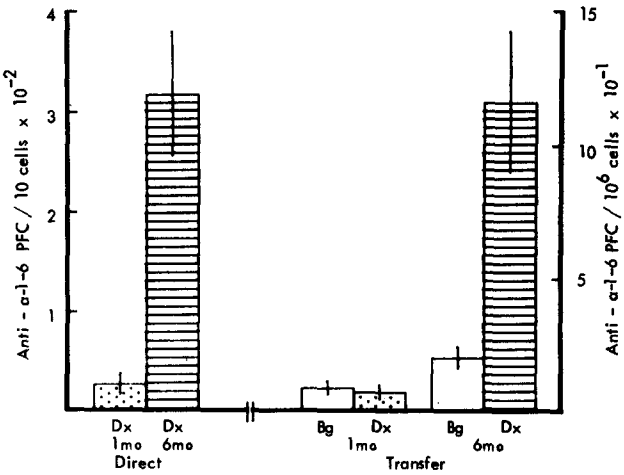

FIG. 3. Inability of spleen cells from young A × CBA mice to respond to dextran directly after immunization (left part) or after transfer into adult (3 mo) lethally irradiated syngeneic recipients (right part). 1 or 6 mo old mice were immunized with 10 μg of native dextran directly or immediately after transfer of 25 × 10^6 spleen cells into 600 rads irradiated recipients. The response was tested 5 days later. Bg indicates the PFC response in nonimmunized mice.

whereas 6 mo old animals or cells from such animals responded normally irrespective of their environment (Fig. 3).

Unresponsiveness to Dextran in Young Animals is Not Due to Presence of Suppressor or Lack of Helper Cells. The possibility that regulatory mechanisms, such as the presence of suppressor cells or the absence of helper cells (13), are responsible for the failure of young mice to respond to dextran was studied by transferring cells from young or adult donors, either separately or together, into 3 mo old syngeneic irradiated recipients. Five different combinations were used: cells from young mice alone, cells from adults alone, cells from young mice mixed with cells from adults, irradiated cells from young mice plus adult cells and, finally, young cells mixed with adult irradiated cells.

As can be seen from Fig. 4, there was no statistically significant difference in the response when immunocompetent adult cells were transferred separately or together with cells from immature animals. Thus, cells from young animals of dextran high responder strains failed to produce antibodies even after transfer to old responding mice and there was no detectable influence by admixing them with untreated or irradiated lymphocytes from old animals after transfer to irradiated responding recipients (Fig. 4).

Before 1 Mo of Age Mice of High Responder Strains Do No Express Immunoglobulins against the α-1-6 Epitope of Dextran. Taken together, the data shown above indicate that young animals from dextran high responder strains behave in the same way as some α-1-6-nonresponder strains studied in our laboratory (1). These strains failed to express immunoglobulins directed against the α-1-6 epitopes. The possibility that young animals are nonresponders to dextran B512, because they fail to express V genes against the α-1-6 epitope was tested in vitro by the use of a polyclonal B-cell activator (LPS)

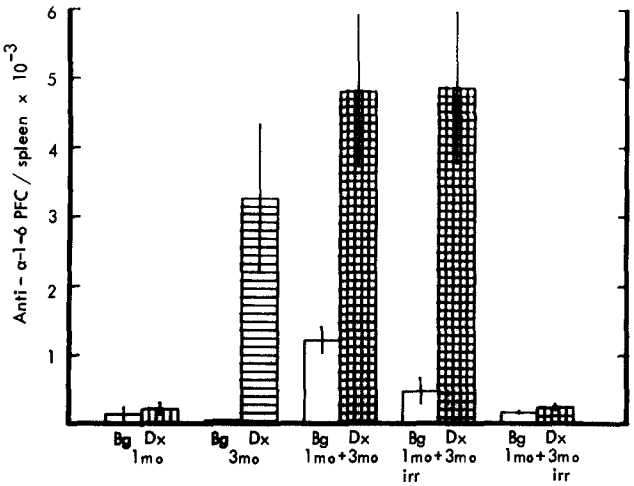

FIG. 4. Absence of interaction between spleen cells from young and adult CBA mice in the response to dextran after transfer into lethally irradiated adult syngeneic recipients. 40 × 10^6 Spleen cells from 1 mo old mice were transferred alone or together with an equal number of cells from 3 mo old mice into the irradiated recipients with or without an immunogenic dose of native dextran. In addition, transfers were made with mixtures where one part had been irradiated with 6,000 rads (indicated by "irr"). The immune response was tested 5 days after transfer. Bg indicates response in nonimmunized animals.

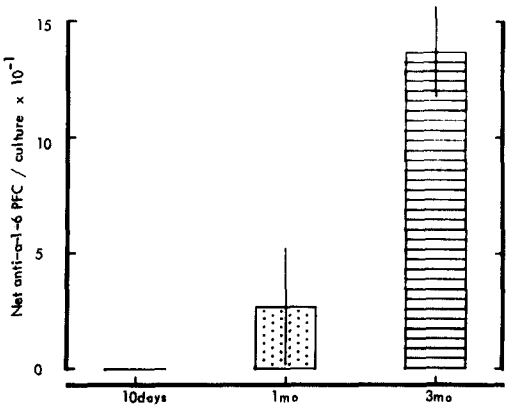

FIG. 5. Ability of polyclonal concentrations (100 μg/ml) of LPS to induce anti-α-1-6 PFC in spleen cell cultures of spleen cells from B10.5M mice of different ages. Serum-free high density cultures were employed and the response was tested after 2 days in culture. Only net PFC per culture is indicated.

known to be able to reveal the total V gene repertoire of the responding cells (14). In addition, young mice were immunized with dextran conjugated to the plant protein edestin, a conjugate which is thymus dependent, and which therefore would activate potentially responding thymus dependent anti-α-1-6 recognizing B cells.

For the in vitro experiments with LPS both high and low density culture systems were used. No significant anti-α-1-6 response could be detected when mice younger than 1 mo were tested in either high (Fig. 5) or low (Fig. 6) cell

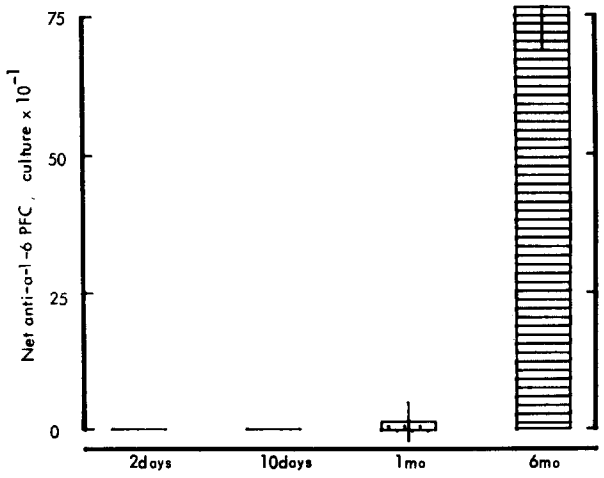

FIG. 6. Same experiment as in Fig. 5, but with low cell density conditions and spleen cells from CBA mice.

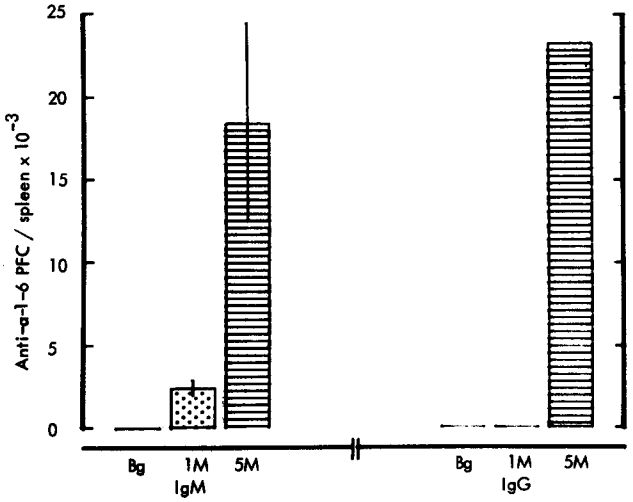

FIG. 7. Development of IgM and IgG PFC against α-1-6 in A × C57BL mice of different ages after immunization with the thymus-dependent antigen edestin-dextran. The mice were immunized twice with 100 μg/animal given i.p. with Freund's adjuvant and the response tested at day 8 after the last challenge. The IgG PFC represents the number of PFC obtained with developing serum minus the number of direct IgM PFC.

density cultures, even though older mice responded in the expected fashion in both culture systems.

For the in vivo experiments, 100 μg of edestin-dextran in complete Freund's adjuvant were given i.p. The animals received two injections of the conjugate and both the IgM and the IgG response against α-1-6 was tested 8 days after the second injection. As can be seen in Fig. 7, young animals did not develop a significant IgM or IgG anti-α-1-6 response, even though edestin was found to be a good carrier for dextran in older animals. Similar results have been found when dextran was coupled to protein A (data not shown).

Discussion

Failure to respond with IgM antibody synthesis to a thymus-independent antigen can be fundamentally caused by two mechanisms (15), namely: (*a*) lack of a subpopulation of B cells having PBA receptors for the PBA property of the antigen or absence of such receptors on B cells. (*b*) Lack of expression of Ig receptors responsible for antigen focussing, either because of lack of the proper V genes or lack of V gene expression.

We have previously described one strain (CBA/N), where unresponsiveness to dextran B512 was caused by the absence of the relevant PBA receptors or a B-cell population possessing such receptors (1). Consequently, CBA/N mice could not respond to dextran or to any hapten coupled to it.

A completely different situation was found with strains on A background, which were also nonresponders to dextran B512 (1). In this case the mechanism of unresponsiveness was due to lack of expression of Ig receptors directed against α-1-6, whereas PBA receptors for dextran were fully functional. The molecular basis for the absence of Ig receptor expression is not known as yet.

The mechanism responsible for unresponsiveness to the α-1-6 epitope of dextran B512 in young animals of high responder strains was analyzed in an analogous way with particular emphasis on the cellular responsiveness to the PBA property of dextran, the presence of Ig receptors against α-1-6 and the possible existence of suppressor cells or lack of helper cells. The unresponsive state to dextran B512 found in young animals from high responder strains was not due to lack of PBA receptors on B cell for the antigen, since dextran was a good carrier for the hapten FITC at the time of development when the animals were nonresponders to the α-1-6 epitope on the same molecule. Direct experiments also showed that dextran was a potent PBA for cells from young animals.

The possibility that suppressor cells existed in young animals or that their environment was suppressive was made unlikely by the findings that cells from 1 mo old mice were unable to produce antibodies against dextran when transferred into lethally irradiated 3 mo old syngeneic recipients. In other experiments both cells from young and old mice were irradiated, a procedure supposed to eliminate suppressor cells (13), and thereafter admixed in various combinations. However, irradiation did not change the results in such a direction that would be compatible with the existence of suppressor cells. Administration of spleen cells from 1 mo old animals together with fully responsive cells from older animals did neither increase nor decrease the number of anti-α-1-6 PFC that was expected from the administration of spleen cells from adult animals alone. The same results can be used as an argument against the possibility that young mice lack some types of helper cells.

Mice under 1 mo of age were complete nonresponders, even when dextran was conjugated to the thymus-dependent protein edestin, which was a good carrier for dextran in adult animals. Finally, the demonstration that LPS at polyclonal concentrations, which normally will induce antibody synthesis against all determinants tested for, failed to activate antibody synthesis against α-1-6 in young animals, indicates that young mice do not express V genes against α-1-6.

When dextran nonresponder strains on A background were studied for the

mechanisms of nonresponsiveness it was conclusively demonstrated that these strains failed to express V genes coding for α-1-6 antibodies (1) and subsequent studies (unpublished observations) showed that unresponsiveness was linked to the V_H chain locus, suggesting that these strains actually lack a V gene coding for antibodies against the α-1-6 epitope. The unresponsive state discussed here cannot be ascribed to lack of V genes against α-1-6, since the animals later develop the ability to respond to the antigen. Therefore, there must exist a regulatory mechanism that determines the time in development when a particular V gene is expressed. Although several theoretical mechanisms exist to explain such a timed expression of a particular V gene, there is little or no evidence for highly specific mechanisms operating at the transcription or translation levels. An immunocompetent cell can now be defined as a cell in which a V-C gene translocation has occurred. It seems possible from our results that the V-C gene translocation can occur late in development. Although a mechanism that regulates the timing of this translocation at the DNA level is unknown as yet it seems plausible that regulation occurs at this level.

There has been much interest in the possible existence of a nonrandom development of different V genes during development. Previous studies (16) have shown that antibodies against different antigens in fetal lamb can be induced at different times and analogous studies have been carried out in mice (17). A main problem with these studies is that there may be multiple causes of a delayed detectable appearance of antibodies against a particular antigen, such as different sensitivities of the assay method for various antigens, the unknown dependency of helper cells, such as T cells and macrophages, for expression of immunity. This makes it difficult, if not impossible, to distinguish between maturation of functional versus genetic events. For instance, a thymus-dependent antigen may fail to induce antibody synthesis, because the T cells have not matured to such an extent that it can help in B-cell activation, even though both B and/or T cell may have expressed the V gene against the epitope studied. Even with thymus- and macrophage-independent antigens, it may be difficult to safely distinguish between maturation of Ig and PBA receptors.

The α-1-6 epitope has the advantage that the response to it is mono- or pauciclonal and is genetically linked to the V_H locus. This together with the unique ability of PBAs to reveal the total V gene repertoire of responding B cells makes it possible to safely conclude that the V gene against α-1-6 is not expressed until 30–40 days after birth. Therefore, it seems impossible to deny the existence of (a) mechanism(s) that regulates V gene expression (possibly operating at the V-C gene translocation level) during development.

Summary

Young mice of dextran high responder strains were found to be complete nonresponders to the α-1-6 epitope of dextran during 30–40 days after birth. They also failed to respond to thymus-dependent dextran-protein conjugates. Cells from young and adult mice were activated equally well to polyclonal antibody synthesis by the polyclonal B-cell-activating property of dextran. There was no age difference in the immune response to haptens conjugated to dextran,

indicating that dextran can function as an efficient carrier also in young mice. Unresponsiveness could not be attributed to suppressor T cells or to a suppressive environment in young animals, as shown by transfer experiments, in which living or irradiated cells from young and adult mice were admixed in various ways and transferred to irradiated recipients of different ages. Cells from young mice did not affect the response of adult cells (and the reverse), nor did the age of the irradiated recipient influence the response.

When lymphocytes from young and adult mice were polyclonally activated in vitro by lipopolysaccharide, only cells from young mice failed to synthesize antibodies against the α-1-6 epitope of dextran, although they produced antibodies of all other specificities tested for. It was concluded that young animals fail to express immunoglobulins directed against the α-1-6 epitope during the first 30–40 days after birth. Since the mice possess the V_H gene coding for antibodies against this particular epitope, it was concluded that the timing of V gene expression is regulated during development, possibly at the V-C gene translocation level.

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