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Immune response against two epitopes on the same thymus-independent polysaccharide carrier

1. ROLE OF EPITOPE DENSITY IN CARRIER-DEPENDENT IMMUNITY AND TOLERANCE

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Summary. Immunogenicity and tolerogenicity of two epitopes (α 1-6 and FITC) on the same dextran B 512 carrier were investigated. The following conclusions were made: (1) Both epitopes were thymus-independent and immunogenic and tolerogenic as well. (2) The marked dose differences between the two epitopes with regard to tolerance induction were found to be a consequence of the affinity and the heterogeneity of the responding B cells as well as the epitope density employed for detecting the PFC in a predictable way. (3) The α 1-6 response, in contrast to the anti-FITC response, was homogeneous and of low affinity and the number of precursor B cells was low. (4) Different mouse strains were found to be high-, low- or non-responders to α 1-6, but all the strains tested responded to the FITC epitope coupled to dextran. (5) Dextran and FITC-dextran were polyclonal B cell activators in the strains tested, irrespective of their ability to respond to the α 1-6 epitope.

The findings indicate that epitope density and mol. wt of the immunogen as well as Ig receptor affinity for the epitope on the B cells are variables which markedly influence the binding of the immunogen to the specific B cells and therefore affect the delivery of the non-specific triggering signal.

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INTRODUCTION

Native dextran B512 has a mol. wt of 10–100 × 10^6 and is a thymus-independent (TI) antigen as well as a polyclonal B-cell activator (PBA) (Coutinho, Möller & Richter 1974a). Antigenicity and mitogenicity decrease in parallel with decreasing MW. Another dextran from the B1355 strain of *Leuconostoc mesenteroides* (native B 1355, MW approximately 40 × 10^6) has also been extracted. In contrast to Dx B 512, which is composed exclusively of α 1-6 linkages, this Dx has a branched structure composed predominantly of two types of epitopes: α 1-3 linked glucose residues (35%) and α 1-6-linked glucose residues (57%).

The immunogenic and tolerogenic properties of these two B 1355 epitopes have been studied recently by Howard, Vicari & Courtenay (1975a, b, c). They showed that the epitopes exhibited different immunological characteristics with respect to induction of immunity and tolerance. Thus, it was claimed that α 1-6 was less immunogenic and more tolerogenic

than α 1-3, even though both epitopes were present on the same carrier.

These results are in apparent contrast with the predictions of the one non-specific signal concept (Coutinho & Möller, 1974) which postulates that only the polyclonal B-cell activating properties of the carrier determine immunogenicity in TI immune responses.

In this paper we have created a dual epitope structure on Dx B 512 by introducing the epitope fluorescein iso-thiocyanate (FITC), at a ratio of one molecule of FITC for every 200 glucose residues. We will describe the immunogenic and tolerogenic properties of haptenated dextran with particular attention focused on the affinity of the immune response against the α 1-6 linkage *vs* the FITC hapten. The results are compatible with the prediction of the one non-specific signal hypothesis, but illustrate certain peculiar immunological features of the immune response to dextrans.

MATERIALS AND METHODS

Mice

Mice of the following strains were used in the present study: A/Sn, A.CA, Balb/c, CBA, C57BL and B10.5M and certain hybrids between these strains.

Antigens and polyclonal activators

Native Dx from *L. mesenteroides* B512 (average MW 10–100 × 10^6), was kindly provided by Dr Richter, Pharmacia, Uppsala, Sweden.

FITC-Dx native, was synthetized from Dx native B512 by reacting it with fluorescein iso-thiocyanate (FITC), and was provided by Dr de Belder. The final conjugation ratio was one molecule of FITC for every 200 glucose residues. In addition, commercial preparations of FITC-Dx with mol. wts of 150,000, and 40,000 were obtained from Pharmacia, Uppsala, Sweden.

Lipopolysaccharide from *E. coli* 055:B5 was prepared by phenol water extraction by Professor T. Holme (Department of Bacteriology, Karolinska Institutet).

Con A and PHA were obtained from Pharmacia, Fine Chemicals AB, Uppsala, Sweden and Wellcome Reagents Ltd, Beckenham, respectively.

Assay of antibody synthesis

Anti-Dx α 1-6 plaque-forming cells (PFC) were detected by a direct PFC assay (Jerne & Nordin, 1963) using SRC sensitized with stearoyl Dx B512 with a mol. wt of 70,000, as described before by Howard *et al.* (1975a). We studied different affinity anti-α 1-6 PFC by the method of Pasanen & Mäkelä (1972) by the use of different concentrations (4, 40 and 400 μg) of stearoyl Dx in 10 ml of a 5% suspension of SRBC, in order to detect high, medium and low affinity PFC, respectively. The last concentration proved difficult to use, because the red cells often agglutinated.

Anti-FITC PFC

The coupling of FITC to RBC has been described before (Möller, 1974). 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.

Anti-NNP PFC

RBC were coupled with NNP as described by Pasanen & Mäkelä (1969). These cells were used to detect polyclonal antibody synthesis (Bullock & Möller 1972).

Measurement of DNA synthesis

A microculture technique was used as described before (Coutinho & Möller, 1973). The cultures were serum-free and were assayed on day two by the addition of ^{3}H-labelled thymidine (The Radiochemical Centre, Amersham), with a sp. ac. of 5 Ci/mM. One microcurie was added to each well and the cultures were harvested 24 h later by a multiple cell culture harvester from Skatron AB, Norway.

Induction of polyclonal antibody synthesis

Spleen cells were cultured serum-free (Coutinho & Möller, 1973) in 3-cm diameter plastic petri dishes (NUNC, Denmark) with a cell concentration of 10^7 cells/ml in 1 ml medium (Mishell & Dutton, 1967) set up in triplicate. The cultures were incubated at 37° in plastic boxes filled with a mixture of 10% CO_2, 83% N_2 and 7% O_2.

RESULTS

High and low responder strains to Dx B512

Dx B512 is considered to be a thymus-independent antigen and as expected from this property, it is a polyclonal B-cell activator capable of causing

Table 1. Mitogenicity of native Dx B512 and of native FITC-Dx in serum-free microcultures

Mitogen and dose (μg/ml)	Strain CBA c.p.m.		Strain Balb/c c.p.m.	
	Expt 1	Expt 2	Expt 1	Expt 2
Bg	10,467±679	22,718±925	3,368±43	5,557±51
PHA 1	64,119±484	115,707±4,158	95,776±1,276	104,477±1,940
Con A 1	71,108±1,006	134,684±2,540	121,503±2,217	103,925±6,602
Dx 5.000	44,173±606	81,313±387	13,222±112	19,257±533
FITC-Dx 50	15,695±960	41,255±1,402	7,892±459	7,947±199
LPS 100	43,317±1,013	115,447±2,340	18,518±1,800	20,367±5,996

increased DNA synthesis in B lymphocytes (Coutinho *et al.* 1974a). We also found that Dx was capable of inducing specific antibody synthesis in thymectomized lethally irradiated mice reconstituted with anti-theta-treated bone marrow, even though these mice failed to respond to the thymus-dependent antigen SRC. The same dextran preparations also caused increased thymidine incorporation (Table 1) and induction of polyclonal antibody synthesis (see later). As a polyclonal activator of antibody synthesis, Dx was very efficient and in many experiments as potent as LPS.

It has been reported that high and low responder strains exist to the α 1-3 determinant on dextrans (Blomberg, Geckeler & Weigert, 1972), whereas no studies have been made with regard to the α 1-6 determinant. We found that the strains CBA, B10.5M, C57BL, C3H and others were high responders to α 1-6, whereas A and Balb/c were low responders (compare Figs 2 and 3). Strain A.CA was found to be a non-responder. The analysis of the latter phenomenon will be made in a separate communication.

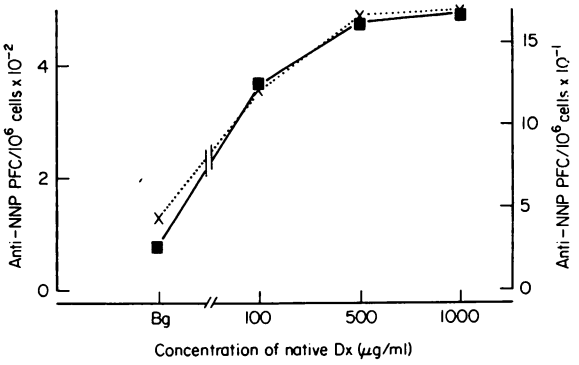

Figure 1. Induction of polyclonal antibody synthesis by native Dx in C3H/Tif (■—■) and A.CA (×.....×) mice. The cells were cultivated serum free in the indicated concentrations of Dx and the response measured at day 2 against NNP-coated SRC. The left scale refers to C'3H/Tif and the right to A.CA.

The cause of the low- or non-responsiveness of the strains mentioned above was not due to a lack of susceptibility to the PBA property of Dx, since all the strains responded polyclonally to Dx, including the non-responder strain A.CA (Fig. 1). The relative magnitude of the Dx induced polyclonal response varied between different strains, but there was no clear correlation between low immune responsiveness to Dx and weak PBA activation. Actually the Dx induced polyclonal activation was similar in different strains when the response over background was considered (Table 1 and Fig. 1).

The specific immune response to α 1-6 induced by native Dx *in vivo* as well as the polyclonal response *in vitro* consisted entirely of IgM PFC and there were no detectable indirect PFC, in agreement with previous findings (Howard *et al.* 1975a, b, c).

Affinity of the anti-α 1-6 response in high and low responder strains

As a first step to analyse the affinity characteristics of the response to the α 1-6 epitope of Dx B512, the immune response in high and low responder strains injected with various doses of native Dx was tested on SRC targets coated with different concentrations of stearoyl Dx. In hapten systems this method has been found to give valid approximations of the range of affinity classes among the PFC appearing after immunization (Pasanen & Mäkelä, 1969). A high epitope density detects low and high affinity PFC,

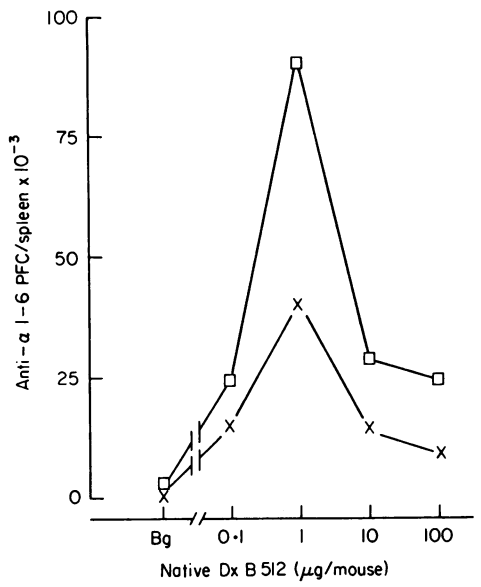

Figure 2. Direct PFC response at day 5 in CBA mice after immunization with Dx native B512. (□——□) Low affinity anti-1-6 PFC, (×——×) high affinity anti-α 1-6 PFC.

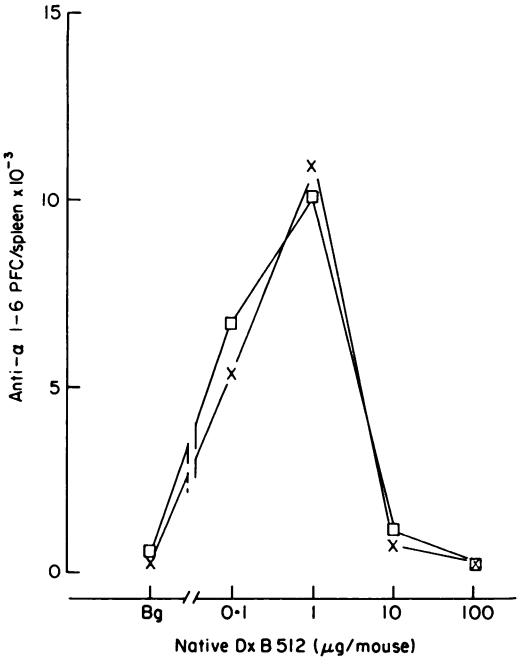

Figure 3. Direct PFC response at day 5 in Balb/c mice after immunization with Dx native B512. (□——□) Low affinity anti-1-6 PFC, (×——×) high affinity anti-α 1-6 PFC.

whereas lower hapten densities preferentially reveals high affinity PFC.

Mice of the strains CBA and Balb/c were injected i.v. with a range of doses (0·1–100 μg) of native Dx and their specific anti-α 1-6 response tested 5 days later using SRC coated with 4 or 40 μg in 10 ml of stearoyl Dx, the former epitope density detecting high affinity PFC.

As can be seen from Figs 2, 3 and 4 the magnitude of the PFC response increased up to the optimal concentration (1 μg) in both high and low responder strains. Subsequently there was a pronounced high dose unresponsiveness in both strains, which occurred after 10 and 100 μg of Dx. It is noteworthy that the high dose unresponsiveness occurred at the same concentrations, regardless of the epitope density on the target cells. This is an unexpected finding as compared to studies in other systems (see also results with FITC-Dx below), where higher concentrations of thymus-independent antigens have been found to be necessary to turn off the response, when the PFC response is assayed with target cells heavily coated with the antigen (Coutinho *et al.*, 1974b).

The number of high affinity PFC to Dx was always much lower than the number of low affinity PFC in the high responder strain CBA (Fig. 2), as expected from findings in other antigenic systems. How-

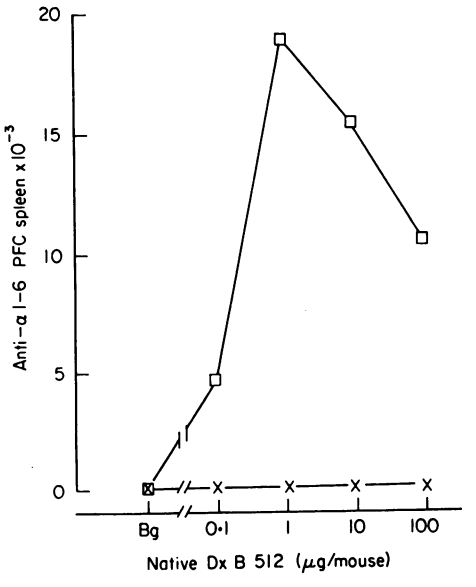

Figure 4. Direct PFC response at day 5 in Balb/c mice after immunization with Dx native B512. (□——□) Low affinity anti-α 1-6 PFC, (×——×) high affinity anti-α 1-6 PFC.

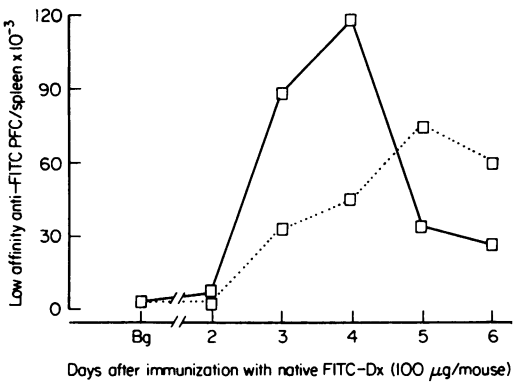

Figure 5. Kinetics of the anti-FITC response after immunization i.v. with 100 μg per animal of FITC Dx native. Low affinity anti-FITC PFC were detected in CBA mice (□——□) and A mice (□ · · · · □).

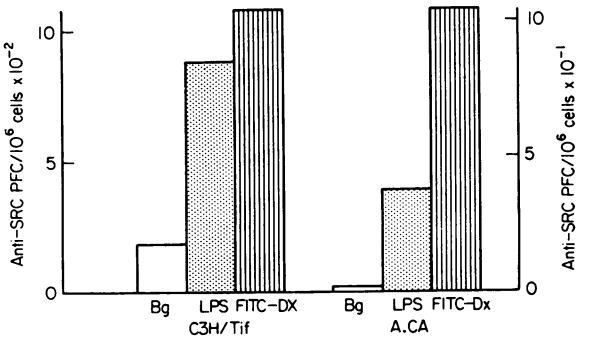

Figure 7. Induction of polyclonal antibody synthesis by 100 μg/ml of LPS (dotted bar) and 200 μg/ml of native FITC Dx (striped bar) in C3H/Tif and A.CA spleen cells. The response was measured after 2 days against NNP coated SRC. All cultures were serum free. The left scale refers to C3H/Tif and the right to A.CA.

ever, the low responder strain Balb/c behaved differently. Thus, the number of high affinity PFC varied considerably from experiment to experiment and was either as high as low affinity PFC, or else—in other experiments—they were nearly undetectable (Figs 3 and 4). It will be shown below that Balb/c mice produce antibodies of very homogeneous affinity characteristics. This is most likely the reason for the number of high affinity cells in Balb/c, since the coupling procedure may vary in efficiency between different experiments and this will cause a variable result in strains that exhibit a homogeneous PFC response.

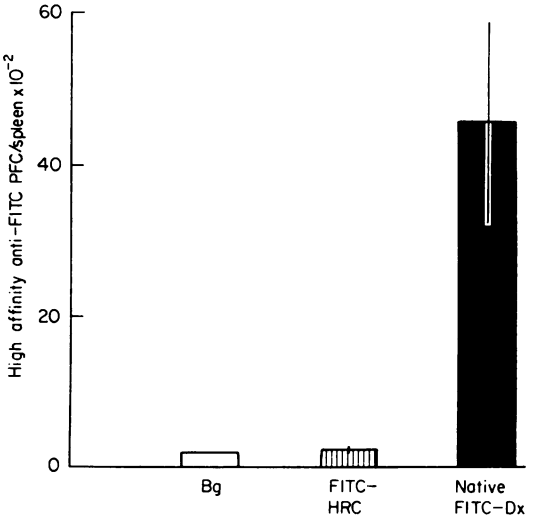

Figure 6. Thymus-independency of the anti-FITC response. FITC-Dx native was injected into thymectomized, lethally irradiated AxCBA mice that had been reconstituted with anti-θ treated syngeneic bone marrow cells. Direct high affinity anti-FITC PFC were measured 5 days after immunization with FITC-HRC (▥); FITC-Dx native (■) and Bg (□).

The anti-FITC response after activation with FITC-Dx

Analogous studies were made with FITC-Dx, in order to investigate whether a different epitope on the same carrier would behave in a similar way. As a first step the kinetics of anti-FITC response were studied in high and low responder strains for the carrier. As can be seen in Fig. 5, the peak response occurred at day 4 in the high responder strain CBA and one day later in the low responder A strain. However, the actual magnitude of the response was usually similar in both strains.

These findings demonstrate that low responder strains to the Dx carrier still can mount a normal immune response to another epitope coupled to the carrier. The FITC epitope on Dx was also found to be a TI antigen (Fig 6).

The mitogenic and PBA properties of FITC-Dx were somewhat reduced compared to unconjugated dextran (Table 1), but the conjugate retained a marked ability to induce polyclonal antibody synthesis, being equal or superior to LPS in several experiments (Fig. 7). Both high and low responder strains to dextran were susceptible to the PBA properties of FITC-Dx. As will be shown below, the

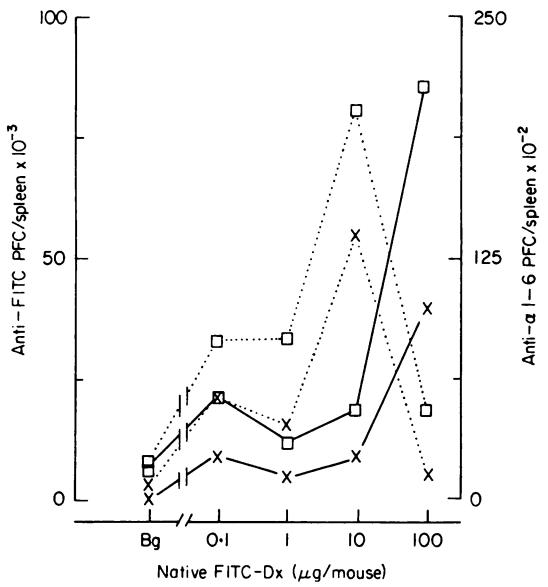

Figure 8. Direct anti-FITC and anti-α 1-6 PFC response at day 5 in CBA mice after immunization with FITC Dx native. Both high and low affinity PFC were detected against both epitopes. (□——□) Low affinity anti-FITC PFC, (×——×) high affinity anti-FITC PFC, (□ · · · · □) low affinity anti-α 1-6 PFC, (× · · · · ×) high affinity anti-FITC PFC.

slightly decreased PBA property of FITC-Dx, as compared to unconjugated Dx, is paralleled by a similar difference with regard to immunogenicity against α 1-6 between the two preparations, as predicted from the one non-specific hypothesis for immunocyte activation.

The anti-α 1-6 and anti-FITC response after immunization with FITC-Dx

High and low responder mice were injected with different doses of FITC-Dx native ranging from 0·1–100 μg/mouse. The immune response against α 1-6 and FITC was determined 5 days later using both lightly and heavily coated target cells. The results are shown in Figs 8 and 9. Apart from the magnitude of the response to Dx, both high and low responder mice gave similar results. Thus, the anti-α 1-6 dose–response curve was shifted, and 10 μg caused the optimal PFC response measuring low affinity PFC, as compared to 1 μg with unconjugated Dx. The high affinity anti-α 1-6 PFC were undetectable in Balb/c and were lower in number in CBA, as was found after immunization with Dx alone. The shift of the dose response is compatible with the decreased PBA property of the FITC-Dx conjugate as compared to Dx, but there is also a reduced availability of α 1-6 epitopes as a consequence of the FITC conjugation (see later).

High dose unresponsiveness to α 1-6 occurred sharply after injection of 100 μg/mouse in both high and low responder strains.

The PFC response to FITC was identical in the two strains studied. The optimal number of PFC occurred after injection of 100 μg/mouse. There was a clear difference between high and low affinity PFC at all doses tested, the high affinity PFC always being fewer.

It is clear from these findings that the FITC epitope on the Dx carrier is much less immunogenic in the sense that the optimal response occurred at a higher dose. However, the number of PFC against FITC was higher (see the Discussion). Since the FITC epitope is conjugated to every 200 glucose residue on the molecule, its lower immunogenicity is expected. Thus, there are considerably more α 1-6 linkages than FITC epitopes on the molecule, which cause a more

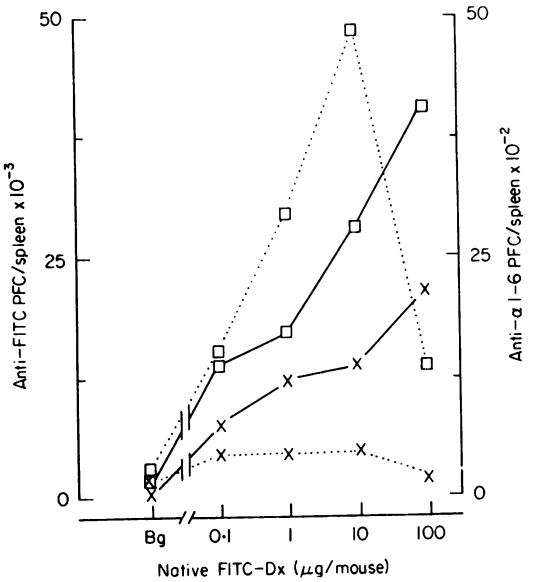

Figure 9. Direct PFC response at day 5 in Balb/c mice after immunization with FITC-Dx native. Both high and low affinity PFC directed against the two epitopes present in the molecule were detected. (□——□) Low affinity anti-FITC PFC, (×——×) high affinity anti-FITC PFC, (□ · · · · □) low affinity anti-α 1-6 PFC, (× · · · · ×) high affinity anti-α 1-6 PFC.

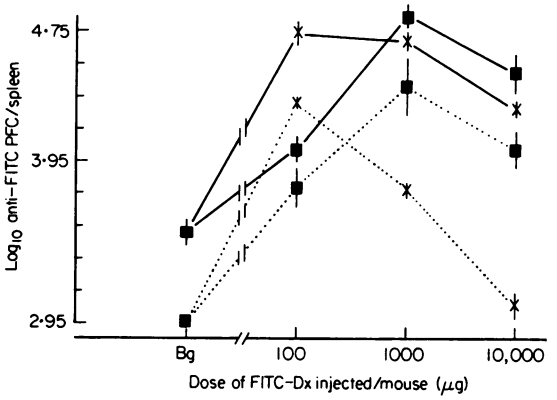

Figure 10. Anti-FITC PFC response at day 4 after injection of different doses of FITC-Dx 150,000 mol-wt (×) or FITC-Dx 40,000 mol-wt. (■) Both high (. . . .) and low (——) affinity anti-FITC PFC were determined.

efficient binding of Dx to the α 1-6 than to the FITC reactive B cells. However, the epitope density by itself is not responsible for triggering or high dose unresponsiveness, as shown before (Möller, Coutinho & Persson, 1975).

Howard *et al.* (1975a, b and c) suggested that two epitopes on the same TI carrier behaved differently: both were immunogenic, but only one tolerogenic. To study whether this was the case in the present test system, FITC-Dx of two different mol. wts (40,000 and 150,000) were injected into mice in doses ranging from 100 to 10,000 μg/mouse and the anti-FITC response tested 4 days later. As can be seen in Fig. 10 all preparations of FITC-Dx caused high dose unresponsiveness. There were several characteristic features of the dose–response curve. (1) High affinity PFC were turned off at lower antigen concentrations than low affinity PFC. (2) High mol. wt FITC-Dx induced antibody synthesis and high dose unresponsiveness at lower antigen doses than low mol. wt FITC-Dx. These two findings are not unexpected, but they emphasize the importance of determining affinity of the response, when comparing immunogenicity and tolerogenicity of a certain antigen.

Thus, high dose unresponsiveness could be induced to FITC as well as the α 1-6 epitope when both were presented on the same molecule. Tolerance to α 1-6 occurred at 100 μg/mouse and there was no detectable difference when the target cells were lightly or heavily conjugated with the epitope, indicating a homogeneous affinity response to this epitope. On the contrary, the FITC epitope required much higher antigen doses for induction of high dose unresponsiveness (10,000 μg) and there were marked dose differences with regard to tolerance induction depending on mol. wt and the affinity of the PFC, as expected from a response involving antibodies having a broad spectrum of affinities for the epitope. As can be seen from the figure, it could erroneously have been concluded that tolerance to FITC could not have been induced, if only PFC of low affinity had been tested.

Affinity of anti-α 1-6 and anti-FITC PFC after immunization with Dx or FITC-Dx

To study directly the affinity characteristics of the PFC against the two epitopes after immunization with FITC-Dx, the PFC were inhibited by the incorporation of antigen or hapten into the agar. AxCBA mice were immunized with 10 μg of native Dx or 100 μg of native FITC-Dx and the spleens were removed after 5 days. The anti-α 1-6 PFC were inhibited by the incorporation of different amounts of native Dx, native FITC-Dx and Dx T20 (20,000 mol. wt) into the agar. The target cells were SRC coated with 40 μg in 10 ml of stearoyl Dx, which detects PFC of medium affinity. The anti-FITC PFC were inhibited in an analogous way by the use of free fluorescein and FITC-Dx and two types of target

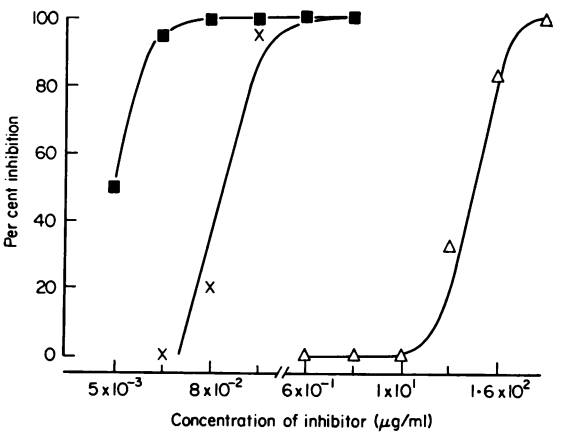

Figure 11. Inhibition of the anti-α 1-6 PFC by incorporation of antigen in the agar during the plaque assay. AxCBA mice were immunized with 10 μg of Dx native B512 and their spleen cells tested at day 5. Different concentrations of the following inhibitors were incorporated into the gel: (■) native Dx B512, (×) FITC-Dx native, (△) Dx B512 20,000 MW. The target SRC were heavily coupled with stearoyl Dx to reveal low affinity PFC.

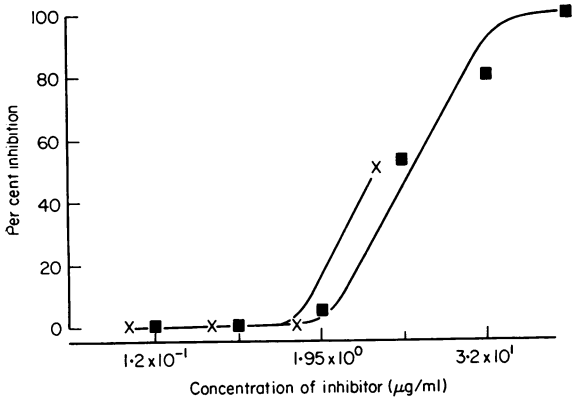

Figure 12. Experiment analogous to that in Fig. 11, but measuring anti-FITC PFC after immunizing mice with 100 μg of native FITC-Dx. Medium affinity anti-FITC PFC were detected after incorporation of different amounts of free fluorescein into the gel (■). In addition, medium affinity anti-FITC PFC were inhibited by native FITC-Dx added to the agar (× ——×).

cells were employed, detecting medium (0·5 mg FITC/ml) affinity PFC. The results are shown in Figs 11 and 12.

It can be seen that the inhibition curve for the anti-α 1-6 PFC is steep, indicating that the PFC are of homogenous affinity. This is in agreement with the conclusions drawn earlier from the dose–response curves for induction of immunity and high dose unresponsiveness. There were marked differences between the ability of the different antigens to inhibit the anti-α 1-6 response. Native Dx was far superior and suppressed 50% of the PFC after addition of about 5×10^{-3} μg/ml in the agar. Native FITC-Dx was less potent requiring the addition of about 8×10^{-2} μg/ml for 50% inhibition. This is in agreement with the previous findings comparing the dose–response curve for induction of immunity of the two compounds and indicates that FITC conjugation has reduced availability of α 1-6 epitopes. Dx T20 was least effective and required about 70 μg for 50% suppression of the PFC.

The inhibition curves for the FITC response indicates a considerable variability of the affinity of the PFC. Target cells coated with a high epitope density required a higher hapten concentration for inhibition than those conjugated with a low epitope density.

Native FITC-Dx was found to be a potent inhibitor in spite of its low content of the FITC epitope. Thus, 5 μg/ml caused 50% inhibition of the PFC. Higher amounts were insoluble. Five micrograms of FITC-Dx contain 0·075 μg of the FITC epitope. This figure should be compared with the dose of free fluorescein needed to cause 50% inhibition (10 μg). Thus, FITC on Dx was much more efficient as an inhibitor than free fluorescein, emphasizing the importance of multipoint binding for stabilizing the interaction between antibodies and the antigen.

Thus, the anti-α 1-6 PFC were of homogeneous affinity, whereas the anti-FITC PFC represented a range of affinity classes. It can also be concluded that the anti-α 1-6 PFC were of lower affinity than the anti-FITC, since the inhibitor Dx T20 with a mol. wt of 20,000 caused 50% suppression after the addition of 70 μg/ml, whereas the hapten FITC with a MW of about 300 caused 50% suppression after addition of 1 μg, even when the target cells were heavily conjugated.

DISCUSSION

These studies show that there exist high-, low- and non-responder strains to the α 1-6 epitope of Dx B512, even though all strains responded approximately equally to the hapten FITC conjugated to Dx. It should be pointed out that only two fundamental mechanisms can explain non-responsiveness to a thymus-independent antigen. (1) The lack of polyclonal B-cell activating receptors on the responding B cells, as is the case of strain C3H/HeJ which fails to respond to LPS and any other determinant coupled to LPS and (2) the absence of Ig receptors in the B-cell population. Since Dx possessed PBA properties of approximately equal strength in all strains tested, and since the FITC epitope on Dx was immunogenic the first alternative is excluded. The second alternative will be analysed in a separate communication. The fact that the anti-FITC response after immunization with FITC-Dx is thymus-independent and that all strains respond to FITC-Dx also argue against a mechanism of non-responsiveness that involves the PBA property of Dx. The magnitude of thymus-independent immune responses should not be correlated with the strength of PBA activity, since the latter is determined by the size of the responding B-cell subpopulation, which expands very little, whereas the former is predominantly a consequence of a pronounced clonal expansion of the antigen-specific precursors.

Howard *et al.* (1975a, b, c) demonstrated that α

1-6 was less immunogenic and more tolerogenic than α 1-3 in Dx B1355. These findings were correctly interpreted to be incompatible with the one non-specific signal hypothesis. However, the findings in the present paper suggest a different interpretation of their experimental results. We have found that both FITC and α 1-6 are immunogenic and tolerogenic, but there were marked differences between the two epitopes in several respects, all of them expected from known properties of the conjugate. Conjugation of FITC to Dx decreased immunogenicity and tolerogenicity to α 1-6, e.g. higher doses were required to induce both of these phenomena, but FITC conjugation also decreased the PBA property. Thus, this finding is compatible with the one non-specific signal hypothesis for immunocyte triggering. The marked differences in doses needed for induction of immunity and tolerance to the α 1-6 compared to the FITC epitope is also expected, since the α 1-6 epitope is continuously repeated in the molecule, whereas FITC is only coupled to every 200th glucose residue. Thus, the molecule will bind much more efficiently to B cells having Ig receptors directed against α 1-6 than against FITC. Even though the antibodies against α 1-6 were found to be of much lower affinity than those against FITC, the great importance of multipoint binding for the stabilization of antigen-antibody interactions will make the anti-α 1-6 reactive cells capable of binding the antigen molecules much more avidly than the B cells recognizing the less frequent FITC epitope. The rationale of this argument is further supported by the influence of mol. wt on the anti-FITC response. An increase of the mol. wt was found to decrease the dose needed for immunization and for induction of tolerance, as expected if efficient binding to the reactive cells is of importance in order for the PBA property of the Dx molecule to deliver the non-specific triggering signal. Further supporting evidence is that high affinity responses were tolerized at lower antigen concentrations than low affinity responses.

The results indicate that there are marked differences in frequency, avidity and heterogeneity of the Ig receptors directed against Dx and FITC. Thus, there appeared to be few anti-α 1-6 precursor cells, because the number of PFC was lower than that to FITC and the precursor cells appeared to have Ig receptors of restricted heterogeneity, since the affinity distribution of the PFC was small. The anti-FITC response was similar to that of conventional antigens, such as haptenated red cells with regard to all these characteristics. Taken together the findings suggest that the peculiarities of the anti-Dx response are to be found at the level of the Ig receptors, rather than in properties related to the triggering mechanism of B cells.

There is an alternative interpretation of the findings of Howard *et al.*, which is supported by the results in the present paper. It appears that they have studied different affinity classes with regard to the α 1-3 and α 1-6 response. Thus, they obtained a 10–100-fold higher PFC response against the α 1-3 as compared to α 1-6 epitope. They also required about 50,000 times more antigen of analogous mol. wt to inhibit the anti-α 1-3 PFC in agar plaque assay as compared to the anti-α 1-6. If they detected low affinity PFC to α 1-3 as compared to α 1-6, it is expected that it would be easier to tolerize the α 1-3 response. We had to inject 10,000 μg of FITC-Dx 150 to cause high dose unresponsiveness to FITC, when the high affinity PFC were detected, but as can be seen in Fig. 10, this dose had a much less pronounced tolerizing effect on the low affinity response. The dose given by Howard *et al.* (1975c) in their attempts to tolerize the α 1-3 response, may have been insufficient, if the PFC were assayed with heavily coated target cells allowing detection of low affinity cells. Even if the conjugation were similar for α 1-3 and α 1-6 their results could still have been due to affinity differences, since the α 1-6 responding cells may have a higher affinity for the epitope, because of its predominance in Dx B1355 or because the Ig receptors against α 1-6 have a higher affinity than the anti-α 1-3.

Taken together the findings show that two epitopes on the same carrier behave similarly with regard to immunization and induction of high dose unresponsiveness. The differences observed with regard to the doses of antigen needed to achieve these effects can all be explained on the basis of affinity differences at the detection level and/or at the B lymphocyte precursor level. However, these differences do not involve the fundamental process of triggering the B cells by the delivery of one non-specific signal, which is an intrinsic property of thymus-independent antigens.

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