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Thymus Dependence of the Immune Response: Response to the Haptenic Determinant NIP in Mice

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Summary. Normal, deprived (thymectomized, irradiated) and reconstituted (thymectomized, irradiated, thymus grafted) CBA mice were immunized with either a polyvalent or a mainly monovalent conjugate of the hapten 3-iodo-4-hydroxy-5-nitrophenylacetic acid and bovine serum albumin. The humoral antibody response to the hapten was determined by the phage inactivation technique.

The response of deprived mice was always significantly lower than the response of either normal or reconstituted animals. However, by increasing the dose of antigen in these mice it was possible to elicit some degree of responsiveness, but only in those groups which had received the polyvalent conjugate.

INTRODUCTION

In mice, the expression of humoral immunity to most antigens so far tested, has been shown to depend upon a population of lymphocytes which have been influenced by the thymus and are therefore known as thymus derived (Leuchars, 1970). Experiments have shown that although both thymus derived and bone marrow derived cells are required for antibody production to sheep red blood cells (SRBC), only marrow derived cells produce detectable antibody (Davies, Leuchars, Wallis, Marchant and Elliott, 1967). The precise function of thymus derived cells in this system remains to be established. However, in relation to the response to hapten-carrier complexes it has been suggested that thymus derived cells are responsible for carrier recognition (Taylor, 1969). Since the response to some of the macromolecular protein antigens used as carriers has already been shown to depend upon the presence of the thymus (Taylor, 1963), it was of interest to discover whether the response to the hapten NIP conjugated to one such carrier was also thymus dependent. The thymus dependence of the anti-hapten response was also tested in relation to variations in hapten density on the carrier molecule.

MATERIALS AND METHODS

Antigen

The hapten used was 3-iodo-4-hydroxy-5-nitrophenylacetic acid (NIP). Two preparations of NIP coupled to bovine serum albumin (BSA) were used for immunization: $NIP_{1.5}BSA$ containing one and a half moles of NIP per molecule of BSA, and $NIP_{34}BSA$ containing 34 moles of NIP per molecule of BSA. NIP coupled to T_2 bacteriophage was used for the antibody assay by the phage inactivation technique (Mäkelä, 1966) and

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NIP coupled to ε-amino-n-caproic acid (NIP-cap, 10^{-6}M) for hapten inhibition. The preparation of NIP and NIP conjugates has previously been described (Brownstone, Mitchison and Pitt-Rivers, 1966).

Animals

Male mice of an inbred CBA strain were used. Mice were either thymectomized at 6–8 weeks of age (Miller, 1960) or left untreated as normal controls. Thymectomized mice were irradiated 1 week after thymectomy with a mean dose of 880 rad at a dose rate of 148 rad/min, from a Siemens Gammatron-1 cobalt unit. Within 2 hours after irradiation all mice received an intravenous injection of 5×10^6 syngeneic CBA bone-marrow cells. Some of the mice were grafted 1 day later with a single lobe of CBA neonatal thymus which was implanted under the left kidney capsule. These mice will be referred to as reconstituted mice. The remaining thymectomized irradiated mice which did not receive a thymus graft will be referred to as deprived mice. The method of preparation of the chimaeras has been described in detail elsewhere (Davies, Leuchars, Wallis and Koller, 1966).

Immunization

All mice were immunized 4 weeks after irradiation. Of the three experimental groups of normal, deprived and reconstituted mice, half of each group received an intraperitoneal injection of 100 μg alum precipitated $NIP_{1.5}BSA$ with 10^9 *Haemophilus pertussis* organisms as adjuvant, and the remaining half received 100 μg alum precipitated $NIP_{34}BSA$ with the same adjuvant, by the same route. Mice were bled at various intervals after immunization and the sera stored at −20° for later study.

Antigen dose in deprived mice

Based on the findings of the first experiment groups of deprived mice (prepared as described above) were injected with either 500 μg, 1500 μg or 5000 μg of alum precipitated $NIP_{1.5}BSA$ with pertussis as adjuvant, or the same doses of $NIP_{34}BSA$, plus adjuvant.

Antibody assay

The phage inactivation technique using NIP-T_2 phage has previously been described (Mäkelä, 1966). The relative proportions of IgG and IgM were determined by the combined 2 ME-reduction-hapten-inhibition method (Kontiainen and Mäkelä, 1967) based on the finding that IgG anti-hapten antibodies are more easily inactivated by free hapten than IgM anti-hapten antibodies, and that IgM antibody activity is abolished by 2-mercaptoethanol (0·2 M). Antibody titres, expressed as the reciprocal of the serum dilution inactivating 50 per cent of the NIP-T_2 phage PFU, were calculated and statistically analysed by the Computing Centre of Helsinki University. In the Tables figures are expressed as $\log_{10}$ values of geometric means ± the standard deviations. All figures have been corrected to two decimal places.

RESULTS

NORMAL MICE

The response of normal mice to NIP following immunization with 100 μg $NIP_{1.5}BSA$ or $NIP_{34}BSA$, differed in both the quality and quantity of the antibody produced accord-

TABLE 1

MEAN ANTI-NIP TITRES OF NORMAL, DEPRIVED AND RECONSTITUTED MICE INJECTED WITH 100 μg ALUM PRECIPITATED $NIP_{1\cdot5}BSA$ PLUS 10^9 *Haemophilus pertussis* ORGANISMS

Day	Experimental group	No. mice	Antibody titre (log_{10})		Per cent IgM
			IgM	IgG	
14	Normal	10	4·15±0·31	4·52±0·58	27
	Deprived	9	3·24±0·48	2·63±0·64	80
	Reconstituted	9	3·97±0·48	3·96±0·78	40
21	Normal	10	4·42±0·37	4·78±0·66	37
	Deprived	6	3·09±0·30	2·28±0·86	65
	Reconstituted	6	3·82±0·59	4·44±0·89	13
28	Normal	9	3·94±0·27	5·00±0·59	11
	Deprived	4	2·99±0·20	2·32±0·11	49
	Reconstituted	5	3·53±0·16	4·78±0·42	5
35	Normal	7	3·80±0·33	4·78±0·65	8
	Deprived	4	3·03±0·35	2·49±0·29	31
	Reconstituted	4	3·27±0·10	4·85±0·06	3
	Uninjected controls	9	2·83±0·37	1·30±0·51	

Figures underlined $P<0{\cdot}001$.

TABLE 2

MEAN ANTI-NIP TITRES OF NORMAL, DEPRIVED AND RECONSTITUTED MICE INJECTED WITH 100 μg ALUM PRECIPITATED $NIP_{34}BSA$ PLUS 10^9 *Haemophilus pertussis* ORGANISMS

Day	Experimental group	No. mice	Antibody titre (log_{10})		Per cent IgM
			IgM	IgG	
14	Normal	10	5·17±0·37	5·05±0·25	50
	Deprived	9	3·32±0·30	1·92±0·22	95
	Reconstituted	10	4·63±0·34	4·60±0·30	46
21	Normal	9	5·09±0·20	5·27±0·55	35
	Deprived	6	3·30±0·18	2·35±0·42	87
	Reconstituted	8	4·42±0·31	4·98±0·34	21
28	Normal	7	4·82±0·26	5·32±0·47	28
	Deprived	5	3·29±0·25	1·67±0.24	97
	Reconstituted	7	4·89±0·40	5·51±0·32	20
35	Normal	7	4·27±0.30	5·05±0·24	13
	Deprived	5	2·77±0·13	1·46±0·26	97
	Reconstituted	7	4·35±0·31	4·43±0·12	43
	Uninjected controls	9	2·83±0·37	1·30±0·51	

Pairs of figures underlined $P=<0{\cdot}001$.

ing to whether the mice were immunized with mainly monovalent ($NIP_{1\cdot5}$) or polyvalent (NIP_{34}) conjugate. The results are shown in Tables 1 and 2. Sera of the mice immunized with $NIP_{1\cdot5}BSA$ had a lower mean total antibody titre (Table 3) throughout the experiment, the peak response occurred at 28 days compared to a 21-day peak response to $NIP_{34}BSA$, which was about twice as high as the peak response to $NIP_{1\cdot5}BSA$.

Since 100 μg of $NIP_{34}BSA$ is more immunogenic than the same dose of $NIP_{1\cdot5}BSA$, the finding that the peak IgM response to $NIP_{34}BSA$ is about five times higher than the peak

response to $NIP_{1.5}BSA$ may not be very significant. Consequently the expression of IgM as a percentage of the total antibody produced at each time gives a more accurate picture of the relative proportions of IgM and IgG in the sera at these times.

DEPRIVED MICE

The response to 100 μg $NIP_{1.5}BSA$ and $NIP_{34}BSA$ was severely depressed in mice in the absence of the thymus. Mean titres of deprived mice were significantly lower ($P<0.001$) than those of normal or reconstituted animals at all times, and generally lower than one

TABLE 3
MEAN TOTAL ANTI-NIP TITRES OF NORMAL, DEPRIVED AND RECONSTITUTED MICE INJECTED WITH 100 μg ALUM PRECIPITATED $NIP_{1.5}BSA$(A) OR $NIP_{34}BSA$(B) PLUS 10^9 *Haemophilus pertussis* ORGANISMS

Day	Experimental group	Total antibody titre ($\log_{10}$)
14	Normal A	4·72 ± 0·47
	Normal B	5·46 ± 0·23
	Deprived A	3·16 ± 0·51
	Deprived B	3·34 ± 0·29
	Reconstituted A	4·37 ± 0·43
	Reconstituted B	4·96 ± 0·24
21	Normal A	4·97 ± 0·51
	Normal B	5·54 ± 0·35
	Deprived A	3·16 ± 0·51
	Deprived B	3·37 ± 0·12
	Reconstituted A	4·68 ± 0·57
	Reconstituted B	5·10 ± 0·30
28	Normal A	5·05 ± 0·54
	Normal B	5·46 ± 0·38
	Deprived A	3·16 ± 0·56
	Deprived B	3·30 ± 0·25
	Reconstituted A	4·81 ± 0·40
	Reconstituted B	5·61 ± 0·33
35	Normal A	4·85 ± 0·57
	Normal B	5·14 ± 0·18
	Deprived A	3·17 ± 0·82
	Deprived B	2·79 ± 0·13
	Reconstituted A	4·86 ± 0·06
	Reconstituted B	4·71 ± 0·17

$\log_{10}$ above the background response of uninjected controls. Figs 1 and 2 show the differences in IgG and IgM antibody titres of normal and deprived mice immunized with $NIP_{1.5}BSA$ and $NIP_{34}BSA$.

The total antibody titres in both groups of deprived mice were rather similar (Table 3), so that the polyvalent conjugate containing a higher density of NIP per molecule of BSA was no more effective in immunizing these mice than the mainly monovalent conjugate, at least not at a dose of only 100 μg of protein.

ANTIGEN DOSE IN DEPRIVED MICE

By increasing the dose of $NIP_{1.5}BSA$ and $NIP_{34}BSA$ in deprived mice, the response to the mainly monovalent conjugate up to a dose of 5000 μg, was no greater than the response

to 100 μg of the same conjugate. The response to the polyvalent conjugate, however, showed an increase of about one $\log_{10}$ in total antibody titre when the dose was increased to only 500 μg; there was a slight further increase at 1500 μg but 5000 μg gave more or less the same response as 500 μg.

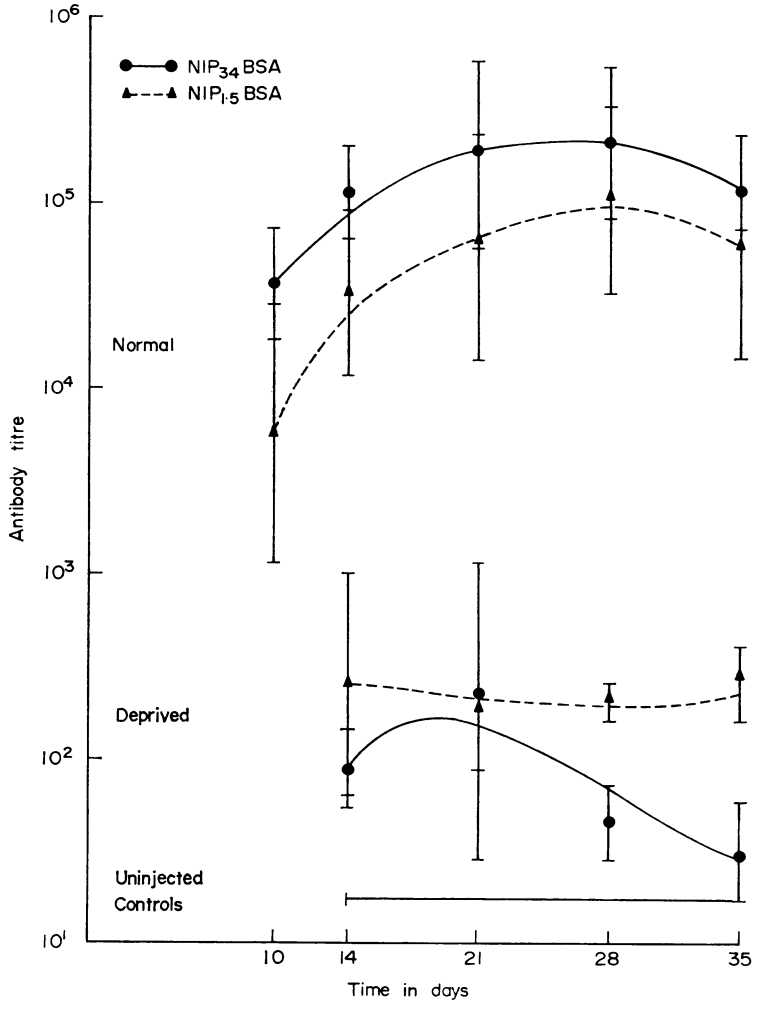

FIG. 1. Mean anti-NIP IgG titres of normal and deprived mice injected with 100 μg alum precipitated $NIP_{1\cdot5}BSA$ or $NIP_{34}BSA$ and 10^9 *Haemophilus pertussis* organisms.

As the results in Table 4 indicate, at 21 days there was a significant difference between the responses to $NIP_{1\cdot5}BSA$ and $NIP_{34}BSA$, at all doses above 100 μg.

RECONSTITUTED MICE

The response of reconstituted mice to both conjugates of NIP-BSA was mostly lower than the response of normal mice, particularly at early times after immunization, but still significantly higher than the response observed in the deprived animals. The peak total

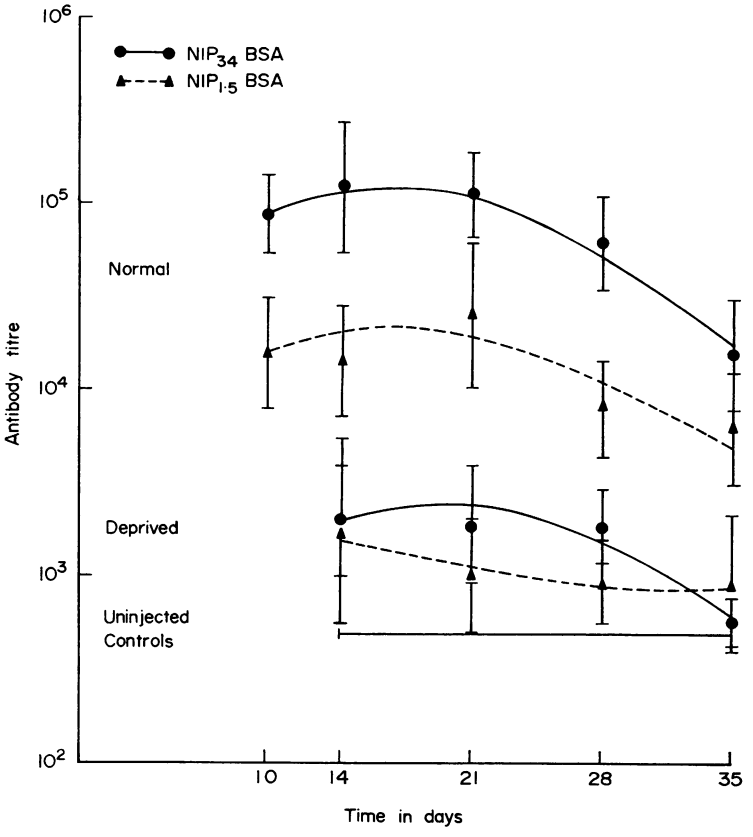

FIG. 2. Mean anti-NIP IgM titres of normal and deprived mice injected with 100 μg alum precipitated $NIP_{1\cdot5}BSA$ or $NIP_{34}BSA$ and 10^9 *Haemophilus pertussis* organisms.

TABLE 4
MEAN ANTI-NIP TITRES OF DEPRIVED MICE 21 DAYS AFTER INJECTION OF VARIOUS DOSES OF ALUM PRECIPITATED $NIP_{1\cdot5}BSA$ OR $NIP_{34}BSA$ AND 10^9 *Haemophilus pertussis* ORGANISMS

	100 μg	500 μg	1500 μg	5000 μg
$NIP_{1\cdot5}BSA$				
IgM titre	3·21±0·30	3·14±0·09	3·42±0·53	3·63±0·25
IgG titre	2·70±0·86	1·60±0·15	2·44±0·88	1·64±0·46
Total Ab. titre	3·40±0·51	3·15±0·09	3·54±0·50	3·64±0·25
$NIP_{34}BSA$				
IgM titre	3·30±0·18	4·10±0·19	4·61±0·71	4·05±0·19
IgG titre	2·35±0·42	2·21±1·26	3·26±0·86	2·24±0·68
Total Ab. titre	3·37±0·11	4·18±0·29	4·64±0·70	4·06±0·20

The difference between all doses of $NIP_{1\cdot5}BSA$ and $NIP_{34}BSA$ above 100 μg (total antibody titre) was significant.

antibody response to both conjugates occurred at 28 days (Table 3) and was little more than half the response of the normal mice in relation to $NIP_{1\cdot5}BSA$, but almost half as high again compared to normal in the mice immunized with $NIP_{34}BSA$. By day 35,

however, the response of the mice which had received $NIP_{1.5}BSA$ was almost equal to the value for normal mice, while the response of the mice which had received $NIP_{34}BSA$ was now only half of the normal value. In these mice there was an even smaller percentage of IgM in the group immunized with $NIP_{1.5}BSA$ than was observed in normal mice, particularly at the late bleedings. On day 35 only 3 per cent of the total antibody produced was attributable to IgM in mice immunized with $NIP_{1.5}BSA$, whereas in the mice immunized with $NIP_{34}BSA$ almost half the total antibody (43 per cent) was IgM.

DISCUSSION

Thymus dependency shows variability both in relation to the responding organism and the nature of the antigenic stimulus. In birds, for example, cells which derive from the thymus are important in cell-mediated immunity whereas responses which involve humoral antibody production are dependent on a population of cells which derives from the bursa of Fabricius (Warner and Szenberg, 1964). In mammals, however, this division of labour amongst cells of the lymphoid series is less well defined, and as the present study illustrates certain humoral antibody responses are very clearly thymus dependent.

Taylor and Wortis (1968) have shown that different immunoglobulin classes produced in a response to SRBC in mice vary in their relative thymus dependency; IgM and IgG_{2a} production were found to be less thymus dependent than the production of IgG_1, when thymectomized, irradiated mice were given increasing doses of SRBC. The results which have been presented here tend to support some of these findings in that higher doses of polyvalent $NIP_{34}BSA$ led to an increase in the response of deprived mice, but only in terms of IgM antibodies.

It has been shown that variations in hapten density on the carrier molecule affect the quality of the antibody produced in relation to the hapten. Polyvalent conjugates, such as $NIP_{34}BSA$, give rise to a higher proportion of IgM than monovalent or mainly monovalent conjugates (Mäkelä, 1970). It may be for this reason, therefore, that the response of deprived mice to $NIP_{34}BSA$ appears to be less thymus dependent than the response to $NIP_{1.5}BSA$. However, it can also be argued that because different numbers of NIP molecules are being introduced with the same amount of protein carrier, then still higher doses of $NIP_{1.5}BSA$ may be required to elicit a response comparable to the response to 500 μg $NIP_{34}BSA$. At present, immunogenically equivalent doses (i.e. doses of the two conjugates which will give the same total antibody titre) have not been fully determined. It still seems valid, however, to predict that the response to the polyvalent conjugate will be less thymus dependent because of the relative thymus independence of IgM antibody production. Since it has been proposed that receptor antibodies on the surface of lymphocytes are similar to the resulting humoral antibodies (Mäkelä, 1970), it is interesting to speculate that the relative thymus independence of IgM production may in some way be related to the antigen focusing mechanism which has been proposed as a function of thymus derived cells (Miller and Mitchell, 1969; Taylor, 1969). In the absence of this device, in deprived mice, it may be possible for 'IgM-like' receptors on precursors of antibody producing cells to concentrate their own antigen by virtue of their large number of combining sites when compared with IgG receptors.

In relation to normal and reconstituted mice the most interesting finding was that these results confirmed those described earlier by Mäkelä, Cross and Ruoslahti (1969), in that there was always a higher percentage of IgM in the mice immunized with the polyvalent

conjugate. In reconstituted mice the responses were more variable and tended to be slightly lower and later than those of normal mice. This apparent weakness may have been due to the combined effects of irradiation and surgery and to the fact that only a single lobe of thymus tissue was grafted into these mice. No satisfactory explanation can be offered for the greater relative difference in the proportions of IgM in the response to both conjugates compared with normal mice. It is possible that inadequate numbers, either, of available stem cells from the bone marrow, and/or of thymus derived cells released from the graft, may in some way have affected the quality of the antibody produced.

There is evidence to show that co-operation takes place between thymus derived and bone marrow derived cells in the humoral antibody response to SRBC (Claman, Chaperon and Triplett, 1966). Recent transfer experiments described by Mitchison (1969) have also provided strong evidence for co-operation between cells of different specificities in the response of mice to hapten-protein conjugates. More recently Raff (1970), using anti-θ serum in a similar transfer system, has shown that the cells responsible for carrier recognition are also thymus derived.

Experiments which have indicated the importance of the carrier in hapten specific immunity suggest that the failure of deprived mice to respond to the hapten, as illustrated here, is due to an initial failure to respond to the carrier.

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