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Effect of carrier priming on antibody avidity in the *in vivo* and *in vitro* immune response*

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Summary. The effect of carrier priming on antibody avidity was investigated under several experimental conditions. Basically, mice were carrier primed with HRBC (horse red blood cells) prior to immunization with TNP (2,4,6-trinitrophenyl) conjugated to HRBC. Immunization was performed either *in vivo* or in spleen cell culture, and avidity of anti-TNP antibodies was estimated from inhibition of direct PFC (plaque-forming cells) by free TNP-BSA (-bovine serum albumin).

The data indicate the appropriate conditions under which carrier priming can enhance antibody avidity. The carrier effect is maximized by priming the animals with 10^4–10^5 HRBC 3–7 days before immunization with a low dose of TNP-HRBC. Hyperimmunization by repeated injections of a high dose of the conjugate does not modify the carrier effect on avidity but it delays the fall of avidity in both carrier primed and unprimed animals. These results are interpreted in terms of T- and B-cell co-operation within the framework of the maturation theory of antibody affinity.

Carrier priming was also found to increase the number of direct PFC of the IgM and, mostly, of the non-IgM classes, a finding in agreement with the notion that T cells can help IgM production and the shift to IgG.

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INTRODUCTION

Hapten-specific antibody response to immunization with a hapten-carrier conjugate can be enhanced by previous priming with the same carrier (Rajewsky, Schirrmacher, Nase & Jerne, 1969).

The carrier effect has been attributed to co-operation between hapten-specific B cells and carrier-specific helper T cells (Mitchison, 1971). Macrophages (Feldmann & Nossal, 1972), as well as humoral factors released from T cells (Schimpl & Wecker, 1972; Doria, Agarossi and Di Pietro, 1972), could participate in this process, whereas anti-carrier antibodies have no effect (Katz, Paul, Goidl & Benacerraf, 1971; Mitchison, 1971).

The question as to whether antibody affinity and immunoglobulin class are affected by carrier priming has been investigated to some extent, but results are contradictory. While Katz *et al.* (1971) found no carrier effect on antibody affinity and Ig class, Hurme, Kontiainen, Seppälä & Mäkelä (1973) showed that carrier priming prior to immunization with the conjugate increased anti-hapten antibody

affinity and accelerated the shift of these antibodies from the IgM to the IgG class. Studies on anti-hapten antibody response and affinity in animals relatively deficient in T cells have provided conflicting results. Gershon & Paul (1971) reported that adoptive transfer of thymocytes into adult thymectomized, lethally irradiated, and bone marrow reconstituted mice resulted in increased anti-hapten antibody affinity. On the contrary, Taniguchi & Tada (1974) found that adult thymectomy in rabbits caused a marked augmentation of antibody affinity, while priming normal rabbits with high doses of carrier diminished affinity. Moreover, Takemori & Tada (1974) showed that adoptive transfer of thymus or spleen cells from carrier-primed donors caused significant decrease of anti-hapten antibody avidity in syngeneic recipient mice.

Thus, it is uncertain whether carrier priming prior to immunization with the conjugate will generate T cells producing helper or suppressor effects on the affinity of anti-hapten antibodies. The present study was undertaken to determine the experimental conditions under which carrier priming enhances antibody avidity in the *in vivo* and *in vitro* immune response. Mice were carrier primed with HRBC prior to immunization with TNP-HRBC. Immunization was performed either *in vivo* or in spleen cell culture. Antibody response was evaluated by counting direct PFC anti-TNP and avidity estimated from PFC inhibition by TNP-BSA. Preliminary results have been presented elsewhere (Doria & Agarossi, 1973; Doria, Agarossi, Di Pietro, Garavini & Mancini, 1974).

MATERIALS AND METHODS

Mice

Three-month-old C3HeB/FeJ and (C57BL/10 × DBA/2)F_1 mice, hereafter designated C3H and BDF_1 respectively, were used in *in vivo* experiments. Only BDF_1 mice were used in *in vitro* experiments. In each experiment all animals were of the same sex.

Red blood cells

HRBC and SRBC were prepared from blood suspended in Alsever's solution, purchased from Sclavo (Italy), and washed repeatedly with PBS (phosphate-buffered saline).

Hapten-carrier conjugates

TNP-HRBC were prepared by heavy coupling of TNBS (2,4,6-trinitrobenzensulphonic acid) to HRBC, as described by Kettman & Dutton (1970), and used as immunogen for *in vivo* or *in vitro* immunization. TNP-SRBC were prepared by light coupling of TNBS to SRBC according to Rittenberg & Pratt (1969), and used as test antigen in the Jerne technique (Jerne & Nordin, 1963) to detect direct PFC anti-TNP. The conjugate TNP-BSA (35 mols TNP/mol BSA) was purchased from Calbiochem (U.S.A.) and used in the Jerne technique to inhibit PFC anti-TNP (Doria, Schiaffini, Garavini & Mancini, 1972). Amounts of the inhibitor TNP-BSA were expressed in terms of TNP-lysyl residues calculated from spectrophotometric measurements of TNP-BSA solutions in PBS at λ max 348 nm by assuming 15,400 as the molar extinction coefficient for the TNP-lysyl residue (Little & Eisen, 1967).

Carrier priming

Prior to immunization with TNP-HRBC, mice were injected i.v. with 0·2 ml PBS containing variable numbers of HRBC as stated in the Results section. Uninjected mice served as controls.

In vivo *immunization*

Normal mice and mice carrier primed 3 days earlier were injected i.v. with 1 ml PBS containing variable numbers of TNP-HRBC, as specified in the Results section. The immunogen was given once in all experiments but one in which mice were repeatedly injected i.v. with TNP-HRBC in 0·5 ml PBS for 10 consecutive days. Assays for direct PFC anti-TNP and antibody avidity were carried out daily.

In vitro *immunization*

Spleen cells from normal mice or mice carrier-primed for a variable time interval as described in the Results section were prepared and cultured as previously described (Kettman & Dutton, 1970). No mercaptoethanol was added to cultures. *In vitro* immunization was performed from day 0 of culture with TNP-HRBC at variable concentrations as indicated in the Results section, while nucleated cell density was $1{\cdot}5 \times 10^7$/ml in all experiments. Cultures were assayed for direct PFC anti-TNP and antibody avidity daily from day 3–7.

Assay for direct PFC anti-TNP

This and the following assay for antibody avidity

have been described in great detail elsewhere (Doria *et al.*, 1972).

Briefly, pooled spleen cells from four mice or four to twenty cultures at a given time of immunization were washed and plated in duplicate dishes with agar containing TNP-SRBC or unconjugated SRBC. Plates were incubated at 37° for 1 h and, after addition of guinea-pig complement, for another 30 min. The mean number of PFC counted in plates with unconjugated SRBC was subtracted from the mean number of PFC in plates with TNP-SRBC to provide the net number of PFC anti-TNP. The magnitude of the anti-TNP response was expressed as the number of direct PFC anti-TNP per spleen or culture.

Assay for antibody avidity

The assay was based on inhibition of direct PFC anti-TNP by soluble TNP-BSA. After the number of PFC anti-TNP per spleen or culture was assessed as described above, the same spleen cell suspension was properly diluted so that an aliquot of it was expected to yield approximately 300 net anti-TNP PFC. This aliquot was plated in duplicate dishes with unconjugated SRBC, or TNP-SRBC, or TNP-SRBC and known amounts of soluble TNP-BSA ranging from 10^{-5}–10^{-1} mg TNP-lysyl residues. PFC were developed, counted and net numbers of anti-TNP PFC in each dish calculated as above. The per cent inhibition of PFC anti-TNP increased as a sigmoid function of the log amount of inhibitor added. From probit analysis of the data and antilog transformations the ED50 (median effective dose of inhibitor that suppresses 50 per cent of PFC) and its 95 per cent confidence limits were calculated by a computer program (Finney, 1962). The reciprocal of ED50 (mg^{-1}) was taken as an estimate of avidity: the higher the antibody avidity, the lower the ED50 and the higher its reciprocal. When avidity was high virtually all PFC were blocked by the greater doses of inhibitor used. When avidity was low, in no instance could a plateau level of inhibition be reached after the addition of very large doses of inhibitor in the non-toxic range. It was assumed that PFC escaping inhibition by the maximum dose of TNP-lysyl residues were cells producing anti-TNP antibodies of extremely low avidity which would be blocked if larger doses of inhibitor could have been used. Inhibition always exceeded 50 per cent, thus covering the most useful range for probit analysis.

Appropriate controls performed as already described (Doria *et al.*, 1972) demonstrated independence of the avidity estimate from the number of plated PFC anti-TNP. Furthermore, when spleen cells from mice immunized with TNP-HRBC were plated with HRBC alone or with HRBC and TNP-BSA ranging from 10^{-5}–10^{-1} mg TNP-lysyl residues no changes in PFC anti-HRBC were observed. This control demonstrates that TNP-BSA was devoid, in the dose range used, of non-specific inhibitory effects on PFC formation which might result from cell toxicity, complement inactivation, or complement consumption by fixation onto complexes of TNP-BSA with anti-TNP antibodies.

Inhibition of direct PFC anti-TNP by anti-IgM antibodies

The relative contribution of IgM-producing cells to direct PFC anti-TNP was determined from inhibition of PFC by guinea-pig anti-mouse IgM monospecific antiserum. Monospecificity of the antiserum was demonstrated by immunoelectrophoresis against mouse whole serum and by its ability to inhibit development of direct PFC anti-SRBC by spleen cells from mice given SRBC 2 days earlier and its inability to develop indirect PFC anti-SRBC by spleen cells from mice given SRBC 16 days earlier, as described in a previous study (Doria *et al.*, 1972) in which the same batch of antiserum was used.

Inhibition of IgM-producing PFC anti-TNP was carried out by plating cells with 0·1 ml antiserum in the Jerne technique (Doria *et al.*, 1972). Cell suspensions were appropriately diluted to yield about 300 PFC when the antiserum was replaced by PBS. It was established in preliminary tests that 0·1 ml antiserum produced maximal inhibition.

RESULTS

Influence of the conjugate dose on the carrier effect

In vivo *experiments*

C3H mice unprimed or carrier primed with 2×10^5 HRBC were injected 3 days later with 4×10^6 TNP-HRBC. Thereafter, four mice of each group were daily killed and their spleens pooled and assayed for anti-TNP antibody response and avidity. The results shown in Fig. 1 demonstrate that carrier priming increases both the number of PFC/spleen and avidity.

In other experiments in which C3H mice received a single injection of different doses of the conjugate

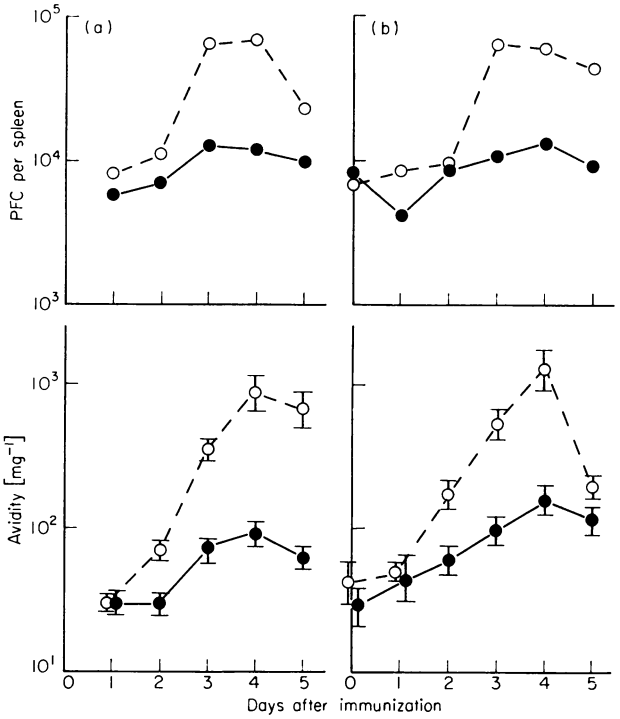

Figure 1. Carrier effect on anti-TNP direct PFC and antibody avidity in C3H mice. Mice unprimed (closed circles) or primed (open circles) with 2×10^5 HRBC 3 days prior to immunization with 4×10^6 TNP-HRBC. (a) and (b) refer to two independent experiments. Vertical bars represent 95 per cent confidence limits of the estimate.

(from 4×10^4–4×10^8 TNP-HRBC), the carrier effect on avidity was more pronounced after immunization with lower doses of the conjugate. Similar results were obtained in BDF_1 mice.

In vitro *experiments*

Two groups each of thirty BDF_1 mice unprimed or primed with 2×10^5 HRBC 3 days earlier were killed, their spleen cells pooled and cultured with different doses of TNP-HRBC. Carrier priming was always found to increase both the number of PFC/culture and avidity. The results reported in Table 1 illustrate that also *in vitro* the carrier effect on avidity can be magnified by lower doses of TNP-HRBC. However, this influence of the conjugate dose on the carrier effect on avidity was found only in five out of ten *in vitro* experiments.

Influence of the priming dose on the carrier effect

In vivo *experiments*

BDF_1 mice unprimed or carrier primed with 2×10^4, 2×10^5, or 2×10^6 HRBC 3 days earlier were immunized with 5×10^4 TNP-HRBC. The results presented in Fig. 2 indicate 2×10^5 HRBC as the optimal priming dose for maximum carrier effect on avidity.

While a carrier dose ten-fold less was almost as effective, a dose ten-fold greater had no effect. In another experiment, in which BDF_1 mice were primed with the same doses of HRBC as above but immunized with 5×10^8 TNP-HRBC, the maximum carrier effect on avidity was also obtained by 2×10^5 HRBC (data not shown). However, the magnitude of the carrier effect was smaller in this experiment with 5×10^8 TNP-HRBC than in that with 5×10^4 TNP-HRBC.

In vitro *experiments*

BDF_1 mice were distributed in six groups each of thirty animals unprimed or primed with a dose of HRBC ranging from 2×10^3–2×10^7 3 days prior to culture. At killing, spleen cells of each group were pooled and cultured with 2×10^5 TNP-HRBC. Both

Table 1. Carrier effect on anti-TNP antibody response and avidity as influenced by antigen dose *in vitro*

2×10^5 HRBC *in vivo* priming 3 days prior to culture	TNP-HRBC ($\times 10^5$) *in vitro*	PFC per culture	Avidity (mg^{-1})
NO	20	1710	200 (283–141)
YES	20	6930	479 (666–344)
NO	2	2210	213 (278–156)
YES	2	5760	890 (1360–583)
NO	0·02	912	162 (223–117)
YES	0·02	1530	788 (1295–480)

Results at day 5 of culture. Figures in parentheses are upper and lower 95 per cent confidence limits of the estimate.

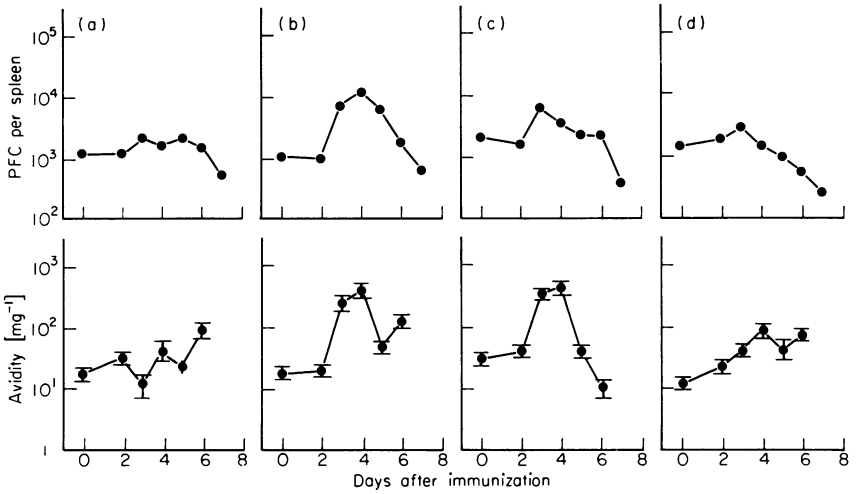

Figure 2. Influence of the priming dose on the carrier effect on anti-TNP direct PFC and antibody avidity in BDF1 mice. Mice unprimed (a) or primed with 2×10^4 (b), 2×10^5 (c), or 2×10^6 (d) HRBC 3 days prior to immunization with 5×10^4 TNP-HRBC. Values at day 0 are from unimmunized mice. Vertical bars as in Fig. 1.

the number of PFC and antibody avidity were increased by carrier priming. Results from one out of two experiments are reported in Fig. 3 and show that the optimal dose of carrier was 2×10^4–2×10^5 HRBC. This finding is in good agreement with the results of the *in vivo* experiments.

Influence of the time of priming on the carrier effect

Only *in vitro* experiments were performed. Thirty BDF_1 mice per group were unprimed or primed with 2×10^5 HRBC and killed after different time intervals, ranging from 1–17 days in separate experiments. Spleen cells were then cultured with $0{\cdot}2 \times 10^5$ or 2×10^5 TNP-HRBC. The results of two experiments reported in Fig. 4 indicate that carrier effect on avidity varied with the time interval between priming and culture: it was maximum at 3 days, declined after 7 days but was still apparent at 17 days. The carrier effect on PFC was similarly influenced

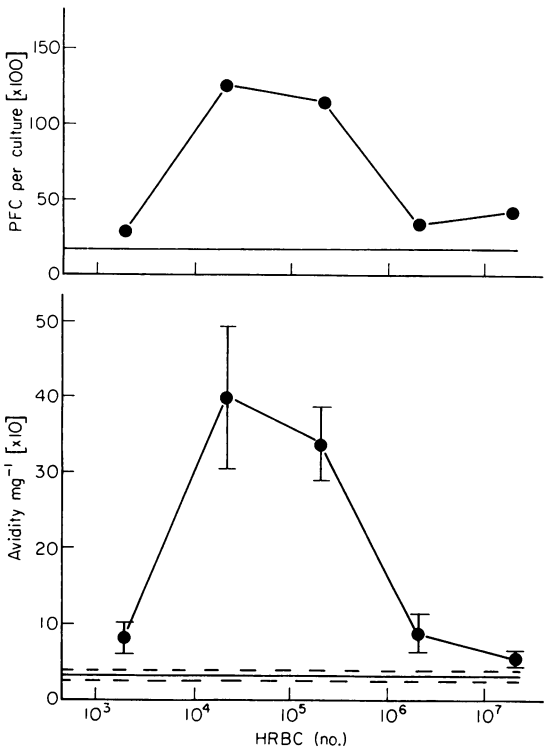

Figure 3. Influence of the priming dose on the carrier effect on anti-TNP direct PFC and antibody avidity in the *in vitro* response at day 5. Spleen cells from BDF1 mice, unprimed (horizontal line) or primed with a single dose of HRBC ranging from 2×10^3 to 2×10^7 3 days earlier, were cultured with 2×10^5 TNP-HRBC. Dotted lines represent 95 per cent confidence limits of the estimate. Vertical bars as in Fig. 1.

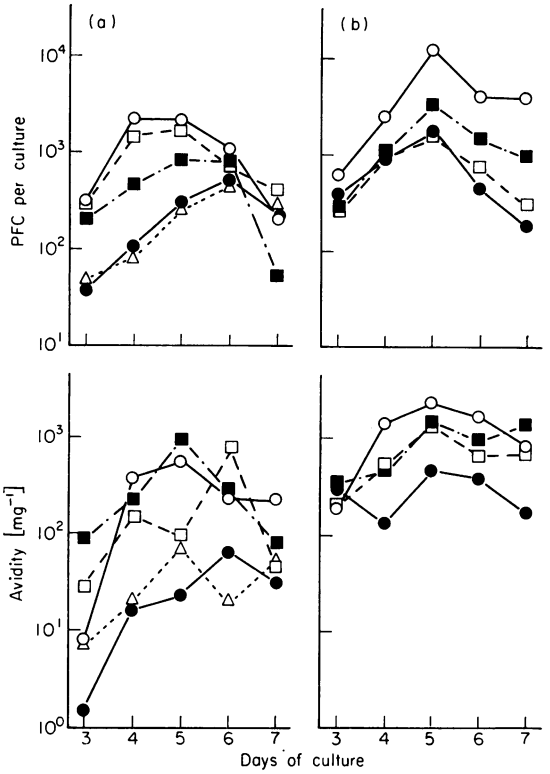

Figure 4. Influence of the time of priming on the carrier effect on anti-TNP direct PFC and antibody avidity in the *in vitro* response. Spleen cells from BDF_1 mice, unprimed (●) or primed with 2×10^5 HRBC prior to culture, were stimulated *in vitro* with 2×10^4 (a) or 2×10^5 (b) TNP-HRBC. Time of priming: (a), 1 day (△), 3 days (○), 7 days (■), 10 days (□); (b), 3 days (○), 10 days (□), 17 days (■).

by the time of priming, since it arose as rapidly but declined somewhat faster than the carrier effect on avidity.

Influence of hyperimmunization on antibody response and avidity

In the *in vivo* and *in vitro* experiments reported above mice or spleen cells were exposed to a single dose of TNP-HRBC. Both PFC number and avidity were found to rise and fall with time sharply, regardless of carrier priming. Whether this pattern, which was previously observed in carrier unprimed mice (Doria *et al.*, 1972), could be modified by hyperimmunization was investigated by the following *in vivo* experiment. C3H mice unprimed or primed with 2×10^5 HRBC 3 days earlier were injected daily with 4×10^8 TNP-HRBC for 10 consecutive days. Assays for PFC and avidity were performed daily from day 1–11 after the first injection of conjugate. Mice were not injected on the day of killing. From the results of Fig. 5 carrier effect on avidity was evident at all experimental times.

The avidity values attained in unprimed and carrier primed mice were of the same order of magnitude as those reached after a single conjugate dose, indicating that the carrier effect on avidity was not modified by hyperimmunization. However, unlike a single injection, hyperimmunization sustained avidity at the maximum level until day 10. The number of PFC per spleen induced by hyperimmunization was much higher as compared to that raised by a single dose, especially in unprimed mice

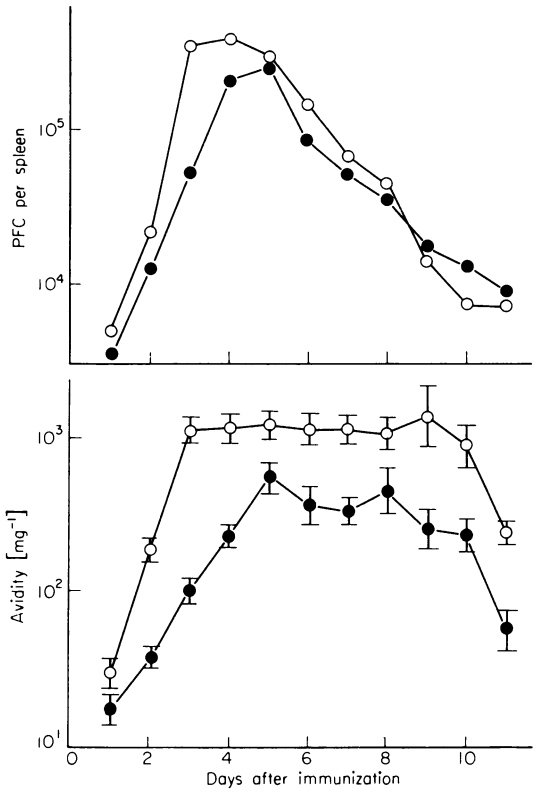

Figure 5. Influence of hyperimmunization on anti-TNP direct PFC and antibody avidity in C3H mice. Mice unprimed (●) or primed (○) with 2×10^5 HRBC 3 days earlier were injected daily with 4×10^8 TNP-HRBC for 10 consecutive days. Vertical bars as in Fig. 1.

in which the number of PFC approached and sometimes attained that in primed mice. Thus, the carrier effect on PFC was very slight during the first 4 days and nil subsequently. As after a single dose of conjugate, hyperimmunization did not change the typical rise and fall of the number of direct PFC in the spleens of both unprimed and carrier primed mice. These findings were confirmed by the results of another similar experiment.

Relative contribution of IgM-producing cells to direct PFC anti-TNP

Spleen cells from BDF_1 mice unprimed or primed with 2×10^5 HRBC 3 days before killing were cultured with 2×10^5 TNP-HRBC. From day 4–7 the per cent of IgM-producing PFC anti-TNP was evaluated in the Jerne assay from plaque inhibition by guinea-pig anti-mouse IgM monospecific antiserum. The results of Table 2 show that both IgM and non-IgM producing cells contribute to direct PFC anti-TNP developed in culture, as previously found for direct PFC anti-TNP developed *in vivo* (Doria *et al.*, 1972). Non-inhibitable PFC (non-IgM) were present on day 4 in both groups but subsequently developed faster in cultures of primed cells. Also the number of inhibitable PFC (IgM) was greater in primed cultures at day 4, 5, and 7, but the carrier effect was less pronounced for IgM than non-IgM at the time of the peak response on day 5 and became inappreciable for IgM on the subsequent day.

Table 2. Effect of carrier priming on Ig class of direct PFC anti-TNP during the *in vitro* immune response to TNP-HRBC

Days of culture	HRBC-priming	PFC/culture			
		Total	IgM	Non-IgM	Non-IgM per cent of total
3	No	326	n.d.	n.d.	n.d.
	Yes	1381			
4	No	561	384	177	32
	Yes	4320	2808	1512	35
5	No	1630	1011	619	38
	Yes	11533	3806	7727	67
6	No	4788	2346	2442	51
	Yes	6310	1767	4543	72
7	No	222	89	133	60
	Yes	2353	682	1671	71

Non-IgM PFC are plaques surviving inhibition by anti-IgM antiserum. n.d. = Not done.

DISCUSSION

The present data show that priming mice with HRBC enhances the subsequent immune response to TNP-HRBC *in vivo* as well as *in vitro*, by increasing the number of anti-TNP antibody-forming cells and the avidity of TNP-specific antibodies. Analysis of the main variables involved in this hapten-carrier system revealed that there are optimal conditions for these carrier effects to occur and to reach maximal levels.

The number of PFC anti-TNP was augmented to a maximum extent by priming mice with 2×10^4–2×10^5 HRBC 3 days prior to immunization. The carrier effect on the number of PFC was favoured by a high dose of TNP-HRBC, both *in vivo* and *in vitro*.

A maximal increase of anti-TNP antibody avidity could be induced by priming mice with 2×10^4–2×10^5 HRBC 3–7 days prior to immunization. The carrier effect on avidity was magnified by low doses of TNP-HRBC, both *in vivo* and *in vitro*.

The rapidity with which the capacity for enhanced anti-TNP antibody avidity was generated as well as the greater efficacy of smaller doses of carrier support the notion that carrier-specific antibodies do not mediate this carrier effect (Katz *et al.*, 1971; Mitchison, 1971). Furthermore, the influence of time and dose of priming suggests that carrier priming induces proliferation or selective recruitment in the spleen of carrier-specific T cells (Sprent, Miller & Mitchell, 1971), low doses and early times selecting cells with helper function (Falkoff & Kettman, 1972), high doses or late times also selecting cells with suppressor function (Taniguchi & Tada, 1974; Gershon, 1974). It should be noticed that suppression as used here is restricted to prevention of the enhancement of antibody avidity, because the avidity values found *in vivo* or *in vitro* upon carrier priming were never below those obtained without priming.

The finding that carrier priming displayed a greater effect on avidity when mice or cells were immunized with lower doses of conjugate suggests that the generation of high avidity antibody-forming cells may depend not only on development of helper T cells but also on selection of hapten-specific B cells with high affinity receptors, as may be expected from the maturation theory of antibody affinity (Siskind & Benacerraf, 1969). Hence, carrier-specific helper T cells may favour antigen selection by expanding the hapten-specific B-cell population on which the conjugate will exert its selective pressure (Katz & Benacerraf, 1972). A variant of this view proposes that high affinity receptor B cells are first selected by antigen and then allowed to proliferate and differentiate into antibody-forming cells if an optimal number of T cells is present (Taniguchi & Tada, 1974). Both hypotheses are supported by our observation that the carrier effect on antibody avidity was more pronounced when mice or spleen cells *in vitro* were immunized with lower doses of TNP-HRBC, which are expected to trigger B cells with higher affinity receptors.

The observed carrier effect on avidity could also be attributed to the helper function of T cells acting as an antigen concentrating device that increases the production of anti-hapten antibodies (Mitchison, 1971). The increased antibody concentration early in the response would lead to predominance of high affinity precursor cells. Indeed, antibodies could compete with cell receptors for antigen so that the decreased free antigen concentration would select higher affinity receptor cells. The selective role of antibodies is sustained by experimental data at the serum (Siskind, Dunn & Walker, 1968) and cellular (Andersson, 1970; And ersson and Wigzell, 1971) levels.

Another mechanism, suggested by Mitchell (1974), ascribes to T cells an antigen clearance function by phagocyte activation resulting in protection of high affinity receptor B cells which are very sensitive to high dose tolerance. Hence, carrier primed T cells should be most efficient in protecting high affinity B cells with consequent increase of antibody avidity when high doses of conjugate are used. However, this expectation is not fulfilled by the present finding of a small carrier effect on avidity when mice were immunized with a high dose of conjugate either once or repeatedly.

In all experiments a concomitant rise and fall of PFC number and antibody avidity was observed during the response regardless of carrier priming. The decline of antibody avidity or affinity with time after immunization has been described previously (Doria *et al.*, 1972; Urbain, Van Acker, De Vos-Cloetens & Urbain-Vansanten, 1972; Werblin, Young Tai Kim, Quagliata & Siskind, 1973). While the rise of avidity is expected from the concept of maturation of the immune response, the fall is more difficult to explain. The appearance of suppressor T cells late in the response may account for the fall of avidity (Tada, Taniguchi & Takemori, 1975). In the hyperimmunization experiment this fall was delayed

probably because a greater number of suppressor cells was required to inhibit a more vigorous response than that following a single immunization.

It is widely accepted that IgG responses require the co-operation of T cells toa greater extent than do IgM responses (Baker, Barth, Stashak & Amsbaugh, 1970; Britton & Möller, 1968; Aird, 1971; Mitchell, Grumet & McDevitt, 1972). However, carrier priming was found to have no influence on anti-hapten antibody class (Katz *et al.*, 1971) or to augment only the IgG response (Miller, Basten, Sprent & Cheers, 1971). The latter finding was referred to a single time after immunization. Our results from an *in vitro* experiment show that both IgM and non-IgM responses were increased by carrier priming, the effect being prevalent for non-IgM at all culture times. These findings *in vitro* confirm previous observations *in vivo* (Kontiainen, 1971; Hurme *et al.*, 1973) and suggest that primed T cells can help the IgM production and the shift to IgG. It cannot be decided whether the observed shift of antibody class was responsible for the increase of antibody avidity. However, since the shift was directed to non-IgM classes of lower valence than IgM it is conceivable that the increase of avidity resulted from a net rise of antibody affinity.

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