Carrier and Hapten Antibody-Producing Cells, *in Vitro* I. Cytokinetics (37563)

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(Introduced by J. Doull)
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In vitro techniques for culturing antibody-producing cells (APC) are useful for determining the effects of drugs on the various developmental stages of the immune response (1–3). By utilizing 2,4,6-trinitrophenyl-substituted horse red blood cells (TNP-HRBC) as the immunogen, the effects of drugs on the development of antibody-producing cells against both carrier (HRBC) and hapten (TNP) can be determined. The development of carrier and hapten antibody-producing cells (APC), *in vitro,* requires the cooperation of T-cells (thymus-derived) and B-cells (bone marrow-derived) (4, 5). When spleen cells from mice previously immunized with the carrier are cultured with the hapten–carrier complex, both T- and B-cells involved in the subsequent *in vitro* anti-carrier response exist in an immunologically altered state, whereas only T-cells involved in the subsequent anti-hapten response exist in an immunologically altered state. Since there is a difference in the immunological status of B-cells involved in the anti-carrier response compared with B-cells involved in the anti-hapten response, these differences should be reflected in the cell kinetics and drug sensitivities of these two responses, *in vitro*. This communication describes the cell kinetics of the *in vitro* immune response obtained by culturing either normal or primed spleen cells with HRBC, TNP-HRBC or without an *in vitro* immunogen.

Materials and Methods. Male C57BL/6 × DBA/2 (BDF_1) mice between 8 and 16 wk of age were obtained from Jackson Memorial Laboratories, Bar Harbor, ME. Sheep red blood cells (SRBC) and horse red blood cells (HRBC) were obtained from Colorado Serum Company Laboratories, Denver, CO. Fetal calf serum was obtained from Reheis Chemical Co., Kankakee, IL. Concentrates of minimum essential Eagle's medium (MEM), Hanks' balanced salt solution, essential amino acids, nonessential amino acids, sodium pyruvate and guinea pig complement were obtained from Grand Island Biological Co., Grand Island, NY.

2,4,6-Trinitrophenyl-substituted erythrocytes (TNP-RBC) were prepared by modifications of the procedure of Rittenberg and Pratt (6) as reported by Pazdernik and Uyeki (7). Cell culture conditions for the induction of APC *in vitro* were described by Mishell and Dutton (8). As a routine procedure, 1×10^7 dispersed spleen cells from normal mice or from mice immunized 3 days previously with 1×10^8 HRBC (primed spleen cells) in 1.0 ml of medium were planted without an *in vitro* immunogen, with HRBC or with TNP-HRBC. Cells were harvested and analyzed on the first, second, third, and fourth days after planting. Details of procedures used to determine hemolysin plaques were reported by Jerne, Nordin and Henry (9). HRBC were used to detect cells producing antibody against the carrier (anti-HRBC response), and TNP-SRBC were used to detect cells producing antibody against the hapten (anti-TNP response).

Spleen cells (1.0×10^7/culture dish) were exposed to 800–1200 R X-irradiation administered at 250 kVp, 0.25 mm Cu as added filtration and a dose rate of 43 R/min.

Results. Antibody-producing cells detected by HRBC or SRBC were absent (0–10 PFC/10^7 spleen cells) when 10^7 unimmunized nucleated spleen cells were assayed; however,

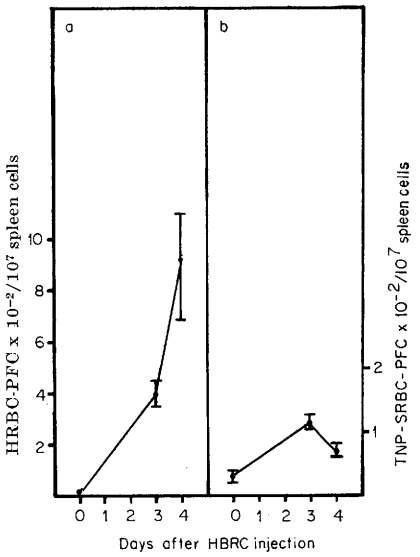

FIG. 1a. HRBC-PFC and (b) TNP-SRBC-PFC per 10^7 BDF_1 spleen cells at various time intervals after injection of 1×10^8 HRBC. Day 0 values represent background PFC from unimmunized mice. Each point represents the mean (± SE) values obtained from three mice.

when TNP-SRBC were used as indicator cells, then (33 ± 2) PFC/10^7 spleen cells were detected.

Injection of mice with 1×10^8 HRBC resulted in the development of an expected primary anti-HRBC response (Fig. 1a) as well as an unexpected and significant increase in the anti-TNP response (Fig. 1b). We refer to this response (Fig. 1b) as a nonspecific *in vivo* enhancement of the anti-TNP response; this response is maximal 3 days after the injection of HRBC.

Both anti-HRBC and anti-TNP responses increased when either normal or primed spleen cells were cultured without an *in vitro* immunogen (Fig. 2). We refer to this response as a nonspecific *in vitro* enhancement of the immune response. The anti-HRBC response, obtained by culturing normal spleen cells without an *in vitro* immunogen, increased from 1.8 ± 0.8 PFC/culture (Day 0) to 53 ± 14 PFC/culture (Day 3) and the anti-TNP response increased from 33 ± 2.0 PFC/culture (Day 0) to 337 ± 26 PFC/culture (Day 3). A different kinetic pattern was observed for the anti-HRBC response when primed spleen cells were cultured without an *in vitro* immunogen. The anti-HRBC response increased from 403 ± 58 PFC/culture (Day 0) to a maximum of 897 ± 38 PFC/culture (Day 1); subsequently, this response dropped to a plateau (Days 2–3) and was followed by a further decline to 87 ± 15 PFC/culture (Day 4). The kinetic pattern of the anti-TNP response, from primed spleen cells cultured without an *in vitro* immunogen, was similar to that observed with normal spleen cells. However, the response was higher at all time intervals reflecting the nonspecific *in vivo* enhancement of the anti-TNP response. The anti-TNP response increased from 115 ± 3 PFC/culture (Day 0) to 844 ± 88 PFC/culture (Day 3).

Figure 3 illustrates *in vitro* immune responses obtained from normal spleen cells cultured with HRBC, TNP-HRBC or without an *in vitro* immunogen. Similar kinetic curves were obtained for the anti-HRBC response when either HRBC or TNP-HRBC were used as the *in vitro* immunogen; a maximum response was observed on Day 4. An

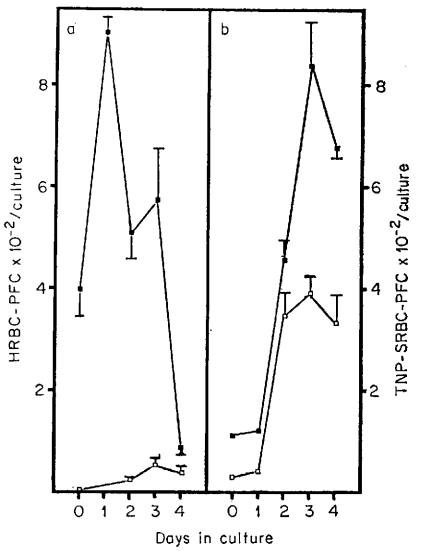

FIG. 2a. HRBC-PFC and (b) TNP-SRBC-PFC per culture when 10^7 BDF_1 spleen cells were cultured without an *in vitro* immunogen. (□—) Normal spleen cells; (■—) spleen cells obtained from mice immunized 3 days previously with 1×10^8 HRBC. Each point represents the mean (± SE) values obtained from duplicate cultures from three mice.

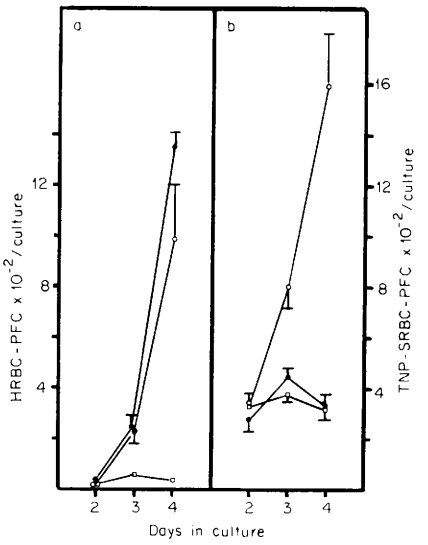

FIG. 3a. HRBC-PFC and (b) TNP-SRBC-PFC per culture when 10^7 normal BDF_1 spleen cells were cultured with: (□—) no *in vitro* immunogen; (●—) HRBC; or (○—) TNP-HRBC. Each point represents the mean (± SE) values obtained from duplicate cultures from three mice.

anti-TNP response greater than the nonspecific *in vitro* enhancement mentioned above was only observed when TNP-HRBC were employed as the *in vitro* immunogen; again, this response was maximal on Day 4.

Figure 4 shows the *in vitro* immune response of primed spleen cells cultured with HRBC, TNP-HRBC or without an *in vitro* immunogen. Again, similar kinetic curves were obtained for the anti-HRBC response when either HRBC or TNP-HRBC were utilized as the *in vitro* immunogen. However, the anti-HRBC response obtained from cultured primed spleen cells differed from the response obtained from cultured normal spleen cells; first, the maximum response occurred a day earlier on Day 3 and, secondly, the maximum response was 60-fold higher than that obtained from cultured normal spleen cells. Again, a significant anti-TNP response above nonspecific *in vitro* enhancement was observed only when TNP-HRBC were used as the *in vitro* immunogen. Like the anti-HRBC response, anti-TNP response also reached earlier peak levels on Day 3 when primed spleen cells were cultured with TNP-HRBC; this peak response was 3-fold higher than that obtained when normal spleen cells were cultured with TNP-HRBC. Also, the anti-HRBC response peaked on Day 3 and sharply declined by Day 4, whereas the anti-TNP response remained elevated (Days 3–4) (see Figs. 4 and 6).

Primed spleen cells (1.0×10^7/culture) were exposed to 800–1200 R of X-irradiation and cultured with TNP-HRBC (Fig. 6) or without an *in vitro* immunogen (Fig. 5). Anti-HRBC and anti-TNP responses were determined at daily intervals. The nonspecific *in vitro* enhancement (Fig. 5) as well as the specific immune response (Fig. 6) was markedly inhibited by X-irradiation.

Discussion. Several models have been proposed in order to explain cellular cooperation (T- and B-cell cooperation) involved in the development of carrier and hapten APC (10, 11). Our results are best explained by a model which assumes that APC are progeny of precursor cells (B-derived cells) of various developmental stages and that each of these stages, including the APC (background

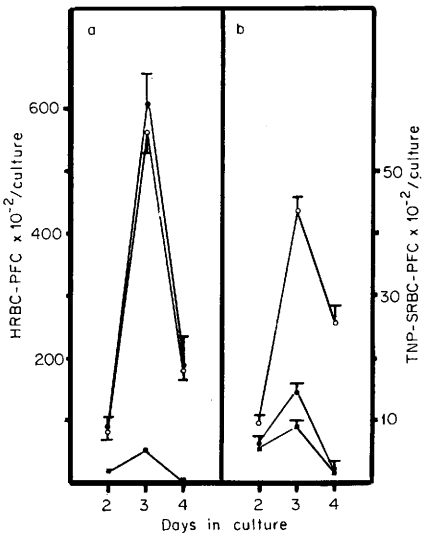

FIG. 4a. HRBC-PFC and (b) TNP-SRBC-PFC per culture when 10^7 BDF_1 spleen cells, immunized 3 days previously with 1×10^8 HRBC, were cultured with (■—) no *in vitro* immunogen; (●—) HRBC; or (○—) TNP-HRBC. Each point represents the mean (± SE) values obtained from duplicate cultures from three mice.

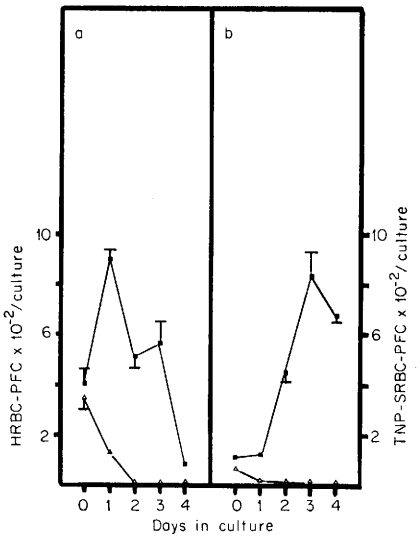

FIG. 5a. HRBC-PFC and (b) TNP-SRBC-PFC per culture when 10^7 BDF_1 spleen cells, immunized 3 days previously with 1×10^8 HRBC, were cultured without an *in vitro* antigen. (■—) Control; (△—) X-irradiated cells. Each point represents the mean (± SE) values obtained from duplicate cultures from three mice.

anti-HRBC, anti-SRBC and anti-TNP PFC), exists in a normal mouse. APC detected on Day 2 require less than 48 hr to mature, APC detected on Day 3 require less than 72 hr to mature, and APC detected on Day 4 require less than 96 hr to mature. Our radiation experiments (Figs. 5 and 6) suggest that the life-span of an APC is relatively short and, hence, most APC assessed on Day 2 probably arose from late developmental cell types, whereas APC assessed late in the *in vitro* immune response (*i.e.*, Day 4) probably arose from earlier precursor cell types. We referred to these various developmental stages in general terms, namely, as early precursor cells (< 4 days to develop), intermediate precursor cells (< 3 days to develop), late precursor cells (< 2 days to develop) and APC (end-stage cells). We envision APC development proceeding through the following maturation sequence: early precursor cells → intermediate precursor cells → late precursor cells → APC. The accessory requirements (*i.e.*, thymic cell synergism, immunogen requirements, time requirements, etc.) may be unique for each developmental stage. This model is compatible with the maturation scheme of APC suggested by Haskill and Marbrook (12). We find this simplified model useful for explaining the kinetics of the carrier and hapten response as well as explaining the effects of immunosuppressive agents on the development of carrier and hapten APC, *in vitro*.

The mechanism of HRBC stimulation of the anti-TNP response is not presently known; a similar enhancement was observed after intraperitoneal injections of Freund's complete adjuvant or keyhole limpet hemocyanin. We referred to this response as a nonspecific *in vivo* enhancement of the anti-TNP response. Our results indicated that maximum levels of APC were obtained on Day 3, suggesting that these APC arose from intermediate → late developmental cell types.

Nonspecific *in vitro* enhancement of anti-HRBC and anti-TNP responses which occurred when normal spleen cells were cultured without an *in vitro* immunogen (Fig. 2) also exhibited an earlier response (markedly enhanced by Day 2 and maximal on Day 3), suggesting that these APC arose from intermediate → late developmental

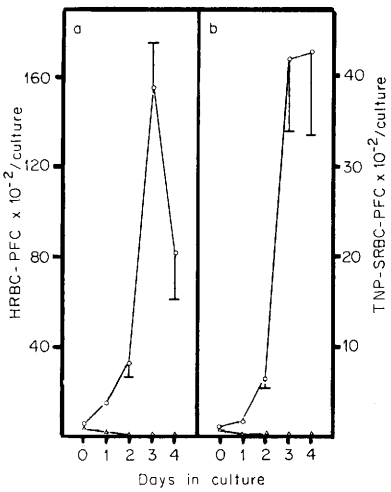

FIG. 6a. HRBC-PFC and (b) TNP-SRBC-PFC per culture when 10^7 BDF_1 spleen cells, immunized 3 days previously with 1×10^8 HRBC, were cultured with TNP-HRBC. (O—) Control; (△—) X-irradiated cells. Each point represents the mean (± SE) values obtained from duplicate cultures from three mice.

cells. Nonspecific *in vitro* enhancement of the anti-HRBC response (Fig. 2a), which occurred when primed cells were cultured without an *in vitro* immunogen, peaked on Day 1; this probably represents a continuation of the *in vivo* response. Nonspecific *in vitro* enhancement of the anti-TNP response (Fig. 2b) exhibited a kinetic pattern similar to that observed with normal spleen cells, except that the magnitude of the response was higher at all time intervals. In all instances this nonspecific *in vitro* enhancement occurred early, suggesting that these APC arose from later developmental cells. Haskill and Marbrook (12) also observed that their more differentiated cell populations could develop into APC without an *in vitro* immunogen. They suggested that fetal calf serum may be responsible for triggering these more highly differentiated cells. Another possible explanation is that washed spleen cells which are cultured, *in vitro,* lack normal feedback control mechanisms.

Similar kinetic patterns were observed for anti-HRBC and anti-TNP responses, when normal spleen cells were cultured with TNP-HRBC (Fig. 3). The maximum response occurred on Day 4, suggesting that the majority of the APC arose from earlier precursor cell types.

When primed spleen cells were cultured with TNP-HRBC, the anti-HRBC response was both accelerated and enhanced (Figs. 4a and 6a) when compared to that obtained with normal spleen cells (Fig. 3a). Likewise, the anti-TNP response was also accelerated and enhanced when primed spleen cells (Figs. 4b and 6b) are compared to normal spleen cells (Fig. 3b). However, the degree of enhancement (anti-HRBC response, 60-fold; anti-TNP response, 3-fold) was considerably less for anti-TNP than for anti-HRBC. Evidence by Fidler, McDaniel and Golub (13) indicated that prior injection of the carrier accelerated the anti-hapten response and this acceleration was mediated by primed T-cells. These results are in accord with the hypothesis that both B- and T-cells are required for APC development. Immunization with the carrier has two effects on the subsequent *in vitro* immune response towards the hapten–carrier complex: (a) immunization with the carrier results in expansion of the T-cell population which results in acceleration and enhancement of both anti-carrier and anti-hapten responses and (b) immunization with the carrier results in expansion and maturation of APC precursors (B-cells) involved in the anti-carrier response; therefore, anti-HRBC responses were accelerated and enhanced to a greater degree than the anti-TNP response.

Summary. Normal spleen cells or HRBC-primed spleen cells were cultured with HRBC, TNP-HRBC or without an *in vitro* immunogen. Anti-HRBC and anti-TNP responses were assessed on Days 2, 3, and 4. Antibody-producing cells detected on Day 2 arose mainly from late, on Day 3 from intermediate and on Day 4 from early precursor cell types. We envision APC development proceeding through the following maturation sequence: early precursor cells → intermediate precursor cells → late precursor cells → APC. The relative proportion of these precursor cell types at any given time is dependent on the immunological status of the animal. Later precursor cell types can be triggered nonspecifically to develop into APC.

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