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Early Changes in T Lymphocytes and Subsets of Mouse Progeny Defective as Adults in Controlling Growth of a Syngeneic Tumor After *In Utero* Insult with Benzo(a)pyrene

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Abstract: Benzo(a)pyrene (BP), a potent carcinogen, severely suppresses the anti-SRBC plaque forming cell response, the mixed lymphocyte response (afferent T cell function), and an *in vivo* graft-vs-host response (efferent T cell function) of mouse progeny exposed to the carcinogen during gestation (11 to 13 days) (Urso and Gengozian, 1984). Immunodeficiency occurs early after birth (1 week) and persists for 18 months. The abnormalities in the T cell mediated responses led us to examine the quantitative profile of T cells and subsets (Lyt 1^+, Lyt 2^+) present in the lymphoid organs during fetogenesis (15 to 19 days) and postnatally. In addition, we examined the ability of 3 to 8 month old progeny and their spleen cells to resist the *in vivo* growth of cells from a syngeneic fibrosarcoma (a tumor that had been induced by BP). Our observations included: 1. Depletion of T cells and subsets in the thymus late (19 days) in gestation and postnatally. 2. Depleted T and Lyt 1^+ cells in the spleen during gestation, while postnatally the former were enhanced and the effect on the latter was variable (enhancement and reduction). 3. In the fetal liver (FL), the T cells were reduced, but the Lyt 1^+ cells were unchanged. 4. The Lyt 2^+ cells were strikingly enhanced in the FL and spleen, but most dramatically for the former. 5. The Lyt 1/Lyt 2 ratio was < 1.00 or $<$ controls in the FL and spleen, a condition which persisted for 30 days postnatally in the latter organ. 6. BP-exposed progeny or their spleen cells were relatively ineffective in resisting *in vivo* growth of transferred tumor cells. These results show that this carcinogenic pollutant induces a marked disorientation of T cells and subsets which can persist for at least 4 weeks postnatally. This suggests disruption of T cell differentiation during ontogenesis which may have profound implications on the ability to resist induction and growth of neoplasias after *in utero* exposure to the carcinogen.

Key words: Benzo(a)pyrene; T lymphocytes; Gestation; Immunodeficient; Lymphoid organs.

ABBREVIATIONS: BP, benzo(a)pyrene; FL, fetal liver; TBDE, trypan blue dye exclusion; CI, cytotoxic index; C', complement.

Introduction

BP, a polycyclic aromatic hydrocarbon and by-product of energy consumption (incomplete combustion), is an ubiquitous environmental pollutant found in diverse industrial (e.g., synfuel production, Griciute, 1979) and domestic sources (e.g., cigarette smoke, Huhti, 1981; automobile exhausts, Grimmer and Pott, 1983; cooking fuel, Aggarwal *et al.*, 1982). The hazardous nature of BP has prompted many investigators to study the biologic consequences of exposure to this agent. Among other physiologic changes, it induces high incidences of tumor (e.g., > 70% in liver and lungs) in progeny when injected parenterally into the pregnant mouse (Bulay and Wattenberg, 1971; Urso and Gengozian, 1980, 1982) or when given only postnatally (Vesselinovitch *et al.*, 1975), and leads to a 30-70% suppression of the anti-sheep red blood cell plaque (antibody) forming cell response in the prenatally exposed progeny (Urso and Gengozian, 1980, 1982) and in adult mice (Stjernsward, 1969). In these studies from 75 to 150 ug BP/g body weight of the animal were used to produce the effects. Further, prenatal exposure to BP and other similiar carcinogens increases sensitivity for tumorigenesis (Nikinova, 1977; Urso and Gengozian, 1982; Tomatis and Parmi, 1971) and for humoral immune suppression with doses comparable to or lower than those given postnatally (Urso and Gengozian, 1982). The immune suppression induced is sustained for the greater part of the animals life span (18 months). We have recently shown that this carcinogen, following prenatal exposure, induces a prolonged suppression (18 months) of cell mediated immunity (30 to 70% of control) as measured by T cell proliferation in the mixed lymphocyte response (afferent function), and by an *in vivo* graft-vs-host response (presumably by T cells) (effector function) (Urso and Gengozian, 1980a, 1983, 1984).

Since it is commonly accepted that the response against tumor involves primarily T cell mediated functions (Baldwin and Robins, 1977; Clark and Harmon, 1980), and that the immune system is programmed to committed T cell lines during fetogenesis (Cantor and Boyse, 1975; Scheid *et al.*, 1975; Murgita and Wigzell, 1981), we decided to analyze the effect of BP on the production of T cells and subsets in the lymphoid organs (thymus, liver and spleen) of mouse progeny both prenatally and during early postnatal life after exposure at midgestation. We also measured the ability of the progeny to inhibit growth of a syngeneic fibrosarcoma (induced by BP) after intravenous transfer of tumor cells, or the capacity of spleen cells from such progeny to affect *in vivo* growth of the tumor in a Winn assay. Briefly, we observed: a) marked reductions in T cells in the FL, fetal spleen and early postnatal spleen, while reductions occurred in the thymus late in gestation and after birth; b) the Lyt 1/Lyt 2 ratio was < the Lyt 2/Lyt 1 ratio in the FL and spleen, indicating increases in Lyt 2^+ cells not expressing detectable Lyt 1 marker in these organs, but not as consistently in the thymus, and c) progeny, or their spleen cells, were deficient in their capacity to inhibit growth of the syngeneic tumor.

Materials and Methods

Animals and treatment

The mice used in these studies were C3H/AnfCum (Cumberland View Farms, Clinton, Tennessee). Mating procedures and exposure to BP were previously described in detail (Urso and Gengozian, 1980, 1982). Briefly, at 11 to 16 weeks of age, 2 females were housed with 1 male and checked daily for vaginal plugs. After the plug was observed (day 0) fetuses were exposed to BP dissolved in corn oil

(experimentals) during midgestation (11 to 13 days) by intraperitoneal injection of the pregnant female with 150 ug BP/g body weight. To avoid injecting the fetus a procedure described by Tomatis and Parmi (1971) was carried out. Briefly, a 26 guage needle (with a 1 ml syringe) was applied into the mid-line of the upper abdomen immediately beneath the peritoneal wall. By this procedure it is highly unlikely that the fetus would be directly exposed to the carcinogen. Polycyclic aromatic hydrocarbons, including BP, or their products, have been identified in the placenta and fetus after administration to the mother by various routes (e.g., Welch *et al.*, 1972; Wunderlich and Tetzlaff, 1973; Alexandrov, 1983). Therefore, the changes that occur in T cells of the progeny (see **Results**) most likely would be due to passage of undesirable products by absorption through the placenta, by suckling, or by products produced in the fetus and/or pups. Fetuses exposed to corn oil only or unexposed progeny served as controls (unexposed animals gave responses similiar to those encountering corn oil).

Quantitative assays

At intervals after injection, the mothers were killed during pregnancy, and the fetuses weighed as were their thymuses, spleens and livers. Mice analyzed postnatally were weighed, sacrificed and their thymuses and spleens also weighed (liver was not studied). The quantity of nucleated cells in the lymphoid tissue was determined by carefully removing the organs, teasing them in tissue culture medium and counting cells in a hemocytometer. Anti-mouse brain associated (θ) antiserum prepared in goats and thoroughly adsorbed with C3H liver and red blood cells was used for the analysis of T cells. It was mixed with lymphoid cells in Eisen's medium in the presence of guinea pig C' (Urso and Gengozian, 1973). For T subset evaluation, lymphoid cells were separated in Cedarlane cytotoxicity medium

(Accurate Scientific) and titrated with monoclonal anti-Lyt 1.1 or 2.1 (Accurate) in the presence of low toxicity rabbit C'. Specificity of these antibodies was determined in several ways including: a) removal of T cells from the spleen by nylon wool fractionation (Julius *et al.*, 1973) and the effluent cells tested with anti-θ antiserum or monoclonal antibodies (>85% of the cells were positive by the TBDE test); b) mixtures containing monoclonal antibodies with rabbit anti-mouse immunoglobulin and mouse thymus cells failed to kill thymocytes as determined by the TBDE test; c) adsorption with syngeneic (Lyt 1.1^+ or Lyt 2.1^+) thymus cells depleted anti-Lyt activity, and d) $C_{57}Bl/6$ thymus cells (Lyt 1.2^+ or 2.2^+) mixed with monoclonal anti-Lyt 1.1 or 2.1 + C' were not killed as determined by the TBDE test.

Several preliminary tests with cells obtained pre-and postnatally from the various organs of normal mice showed no differences in activity between monoclonal anti-Thy 1.2 and anti-θ so that the latter was used for most of the T cell assays. T cell serology was as follows: Cells were incubated with anti-θ diluted 1/10 for 30' at rm temp or on ice (no differences were observed for these incubation methods). After addition of C' the reaction mixture was incubated for 1 hour at 37°C in a water bath. T cells bearing the θ marker were identified by scoring dead cells (those taking up dye) at 10 or 40x magnification using the TBDE test. To identify the Lyt 1^+ or Lyt 2^+ T cells the procedure was to incubate the cells for 1 hour on ice in the presence of monoclonal antibody (final dilution 1/30) followed by an additional incubation of 1 hour at 37°C in the presence of a 1/12 dilution of rabbit C' (method recommended by Accurate). Subsets were identified by the TBDE test as described above. The frequency of cells was calculated from the formula:

$$\% \text{ CI} = \frac{\% \text{ cytotoxic (cells + antibody + C')} - \% \text{ cytotoxic (cells + C')}}{100 - \% \text{ cytotoxic (cells + C')}} \times 100.$$

Initial trials showed that % CI was similar whether C' alone or antibody alone was employed with the cells in the control mixture (e.g., cells + C'). Viability in this mixture was consistently > 90 percent.

Modification of *in vivo* tumor growth

Two kinds of experiments were performed as follows:

1. Winn Assay (Winn, 1961). Five month old normal mice were inoculated subcutaneously in the shaved subscapular region with 10^5 live syngeneic fibrosarcoma cells (0.1 ml) that had been induced by BP. Tumor cells were kindly provided by Drs. R. Snyderman and G. Cianciolo, Duke University and maintained *in vitro* for 10 to 15 passages before use. Viability, tested by TBDE, was always > 90%. Size of the tumor growing *in vivo* was determined by measuring its diameter at right angles with a mm calipers and expressing growth in mm^2. In preliminary dose-response tests (10^4, 10^5, or 10^6 tumor cells), we found that 10^5 cells gave consistent growth (from 10 to 130 mm^2) for up to 30 days. Subsequently, we mixed 10^5 cells with 5 x 10^6 spleen cells from 5 month old experimental or control progeny that had been immunized with 10^6 tumor cells intravenously 15 to 21 days earlier and injected this mixture subcutaneously into the right subscapular region of the syngeneic normal recipients. The growth of tumor at site of injection was measured as described above.

2. Growth of syngeneic tumor in the lungs. The assay was similar to that described by Skov *et al.*, (1976). In preliminary tests we determined the formation of tumor colonies in the lungs of 5 month old normal recipients injected intravenously with varying doses of tumor cells. Lungs were excised at intervals, fixed in Bouin's fluid, and 24 hours later discrete colonies were counted under a

dissecting microscope at 10x magnification. From 7 to 21 days, 10^6 cells led to a progressive increase in colonies (a total of from 4 to 50 when all the lung lobes were scored). With 10^4 cells, fewer colonies (0 to 14) appeared at the same time intervals, while with 10^6 cells by day 10 confluency precluded their identification. Therefore, in all experiments, 10^5 live cells were injected in 3 or 8 month old experimental or control progeny and colonies enumerated on the 21st day after transfer as described above.

Statistical analyses

Differences between the experimentals and controls in cell numbers, T cells and subsets for the lymphoid tissues at intervals analyzed during gestation or after birth, and in the *in vivo* assays for tumor growth, were determined by a one- or two-way analysis of variance and/or by the Student's t-test for unequal sample sizes (Sokal and Rohlf, 1981).

Results

Effect of BP on body weight, organ weight and cell numbers in lymphoid tissue

Following exposure to BP, body weight loss occurred after birth but not prenatally and paralleled the weight loss observed in the thymus and spleen ($p<0.01$ to <0.0001 from 0 to 5 days) (Fig. 1). For the FL, however, weight changes in experimentals were comparable to those of controls. In the thymus, the quantity of cells followed the same pattern as weight loss, i.e., no significant reductions at 15 and 17 days gestation, but at 19 days and later marked decreases occurred

($p<0.01$ to <0.0001) (Fig. 2). In the spleen, however, although cell numbers were significantly lower at postnatal days 1 and 3 ($p<0.001$), for other intervals, the pattern, for the most part, was similar to controls. The amount of cells in FL was also essentially unchanged.

Changes in θ^+ cells and subsets in the lymphoid tissues

Frequency patterns (%CI)

The results depicted in figure 3 show the following: In the thymus a significant reduction of θ^+ cells occurred at 15 days gestation ($p<0.01$), but at the other time intervals the frequency, for the most part, was similar to controls. The frequency for the Lyt 1^+ cells was depressed during gestation and on the first postnatal day ($p<0.01$), while for the Lyt 2^+ cells in general, it was essentially similar to the controls. During fetogenesis in the spleen the frequency of θ^+, for the most part, was dramatically decreased ($p<0.01$ to <0.0001), but increased after birth. A similar pattern occurred for the Lyt 1^+ cells, but the Lyt 2^+ were strikingly elevated ($p<0.01$ to <0.0001). In the FL, the frequency for the θ^+ cells was markedly reduced ($p<0.01$ to <0.0001), unchanged for the Lyt 1^+ cells, and decidedly enchanced for the Lyt 2^+ cells ($p<0.01$ to <0.001).

Absolute changes in θ^+ T cells and subsets

The formula, cell numbers x % CI = number of T cells, was used to calculate the number of θ^+ T cells and subsets in each organ. As shown in figure 4, in the thymus, θ^+ and Lyt 1^+ cells were significantly reduced ($p<0.001$) beginning at 19

days gestation and extending to the third postnatal day, while the Lyt 2^+ subset was diminished only after birth ($p < 0.0001$). In the spleen during fetal life, the Lyt 1^+ subset was below control levels, but showed a striking increase on day 1 after birth ($p < 0.0001$) and was slightly lower than normal on the 3rd day. The Lyt 2^+ subset for this organ was elevated at all intervals tested ($p<0.01$ to <0.0001). For the FL, despite the diminished amounts of θ^+ T cells, the Lyt 2^+ subset was dramatically elevated ($p<0.0001$), while no appreciable change was seen in the Lyt 1^+ cells.

The ratio Lyt 1/Lyt 2 calculated for the thymus of BP-exposed progeny was 1.00 or greater (Table 1). For the spleen and FL this ratio was always <1.00 (from 0.78 to 0.22) ($p<0.01$). This indicates an elevation of T cells bearing the Lyt 2.1 marker but not expressing Lyt 1.1 (or possibly bearing Lyt 1^{+weak} not detectable with the TBDE test). Also, at certain intervals, the θ antigen was expressed in fewer cells than the Lyt marker (Fig. 4), particularly striking in the FL. In one experiment, for the spleen, the Lyt 1/Lyt 2 ratio was either <1.00 or < control up to 4 weeks postnatally (Fig. 5).

In Vivo activity against syngeneic tumor

Spleen cells from BP-exposed progeny (6 month old) showed a depressed ability to inhibit tumor growth (Fig. 6) when compared to controls at the same time intervals. Although an apparent anti-tumor activity was expressed by these cells, it was significantly lower than controls ($p<0.05$ to <0.005). Three and 8 month old BP-exposed progeny also showed a deficiency in their ability to inhibit formation of tumor colonies in the lungs following intravenous transfer of 10^5 tumor cells ($p<0.005$) (Fig. 7).

Discussion

In the experiments reported here we show, unequivocally, that BP, at the dose used, induces severe quantitative disturbances in the T cell population of the lymphoid tissues developing during fetogenesis. In the thymus, a marked depletion of T cells, Lyt 1^+, and Lyt 2^+ cells occurs during late gestation and postnatally. In the spleen, T cells and Lyt 1^+ cells are depleted during gestation, but the former are consistently enhanced after birth. In the FL, the T cells, but not Lyt 1^+ cells are diminished. The most striking change was the elevation of Lyt 2^+ cells in the FL and spleen, but most dramatic for the former.

Since, with this dose of BP, T cell mediated immunity is suppressed at one week after birth, and since ontogeny represents a critical time in the development of the T cell population our purpose was to determine how this damaging dose of the carcinogen affects the quantitative expression of T cells (appearance and distribution in lymphoid organs) during these periods. With comparable amounts, others observed tumorigenic effects in progeny after *in utero* exposure and in virgin mice (See **Introduction**). Further, dose-response studies in adults revealed that the most pronounced immunologic defects occurred at the higher doses (200-400 ug/g body weight, Dean *et al.*, 1983; 40 ug/g, Wojdani *et al.*, 1984). It is important to determine, in our system, the level of sensitivity in humoral and cell-mediated immune parameters and in T cell changes with varying doses of BP and whether the patterns will be similar to those observed by others in adults. In some earlier dose-response experiments (Urso and Gengozian, unpublished), we found that, at 150 ug BP/g body weight, depression of the anti-sheep red blood cell antibody forming cell response in 5 month old progeny was most pronounced (10, 50, and 100 ug were also used). From these considerations, additional experiments of

this nature are in progress and include analysis of T cell behavior during fetal and postnatal life as a function of dose, to provide additional insight into correlations that may exist between tumorigenesis and immunogenesis.

Although the cellular disturbances seen may be coincidental to susceptibility for tumorigenesis, the persistence of abnormal T cell profiles, e.g., increased Lyt 2^+ cells (Lyt 1/Lyt 2 ratio <1.00 or < controls) for at least 4 weeks in the spleen, may reflect the relative immunologic ineffectiveness of these mice (see also Urso and Gengozian, 1984). From our data, the changes in T cell subsets cannot define the immunologic capabilities of progeny later in life. We have evidence that functionally, the Lyt 1^+ and Lyt 2^+ cells of the FL cultured separately do not affect T cell proliferation in a mixed lymphocyte response, while together they dramatically suppress it (Urso and Johnson, 1987). Also, our findings are compatible with those of Stjernsward (1966) who showed that mice, injected with the polycyclic aromatic hydrocarbon methylcholanthrene, were deficient in their response against sheep red blood cell antigens and in their ability to resist *in vivo* growth of a transferred syngeneic sarcoma that had been induced in other mice with methylcholanthrene. It is significant that the effector cells inhibiting growth of a syngeneic fibrosarcoma induced by methylcholanthrene were characterized, in a Winn assay, as Lyt 1^+ cells (Perry and Greene, 1981). Furthermore, Wojdani *et al.*, (1983) and Alfred *et al.*, (1983) demonstrated that spleen cells from adult mice injected with polycyclic hydrocarbons and deficient in killing P815 mastocytoma cells *in vitro* also had sustained decreases of Lyt 1/Lyt 2 ratios suggestive of increased T suppressor cells (these authors, however, did not equate functional activity with a specific phenotype). Finally, we have recently observed suppressor cell activity by spleen cells (presumably T cells) of adult progeny from which the Ia^+ cells were depleted with anti-Ia^k + C', on expression of IgG isotypes by normal B cells in the presence of lipopolysaccharide

(Jones *et al.*, 1986). Although our system needs further characterization, from the data we have described we may surmise that decreased resistance against tumor growth involves T cells. However, participation by macrophages, natural killer cells or other activation mechanisms cannot be excluded. These possibilities are under investigation.

Toxicity by BP is probably not a factor for the changes seen in the spleen and FL. Since the Lyt 1/Lyt 2 ratio is not appreciably changed in the thymus, it is possible that depletion of cells may have occurred from cytotoxic effect by the BP that reaches the gland, e.g., cells giving rise to Lyt 1^+ and Lyt 2^+, or the subsets, are equally sensitive. The presence of BP and similar carcinogens or their intermediate compounds, in the fetal tissues (liver and spleen; the thymus was not analyzed) have been detected as early as 2 to 3 hours after injection of the ^{3}H-labeled compound in the rat (Shendrikova and Aleksandrov, 1974) and at 18 hours in the mouse (the only interval studied) (Wunderlich and Tetzlaff, 1973). However, if a toxic effect does occur it does not become apparent for more than 1 week after exposure to the carcinogen. It is interesting that quantitative deficiency does not take place until late gestation (19 days) and during suckling, after changes have already occurred in the FL and spleen. The significance of this is obscure, but there are 2 intriguing explanations; a) a maternal effect occurs through maternal-fetal exchanges of cellular and humoral elements (Head and Billingham, 1982), and b) the changes reflect disruptions in the FL, an organ which appears to play an integral role in T cell differentiation (Scheid *et al.*, 1975; Stutman, 1978).

We can provide some interpretations of our data in accordance with current proposals concerning ontogenesis of the immune system. In the thymus Lyt 1^+ cells clearly outnumber the Lyt 2^+ cells (see Fig. 4 and Table 1), but in the FL and in the spleen, Lyt 1^-2^+ are increased 2-to 8-fold over the Lyt 1^+2^- cells. One

explanation is that there is a change in the direction of T cell differentiation pathway(s) in these latter organs since the Lyt 1/Lyt 2 ratio remains <1.00, and there is no appreciable reduction of the Lyt 1^+ cells, particularly in the FL. In addition, since the Lyt 2^+ cells of the FL far outnumber those in the fetal thymus (79-fold higher at 15 days and 2-fold at 17 days), it seems unlikely that all the Lyt 2^+ cells in the FL could have migrated to this organ from the thymus. It is possible, however, that many cells may have taken on the Lyt 2 marker extrathymically (i.e., further T cell differentiation in the Fl or other tissue source?). It is interesting that, *in vitro*, 44% of Lyt 2^- embryonic thymocytes (Lyt 1^{+weak}) assume the Lyt 2 phenotype (Ceredig *et al.*, 1983). It is possible, then, that the increase in Lyt 2^+ cells in the FL of the BP-exposed progeny represents extrathymic differentiation of either Lyt 1^-2^- or Lyt $1^{+weak}2^-$ cells in the FL, trafficking out of the thymus *6 times faster* than the controls.

Another interesting observation that may provide relevance to extrathymic cell differentiation during fetal life in the BP-exposed progeny is the reduction in T cells (θ-bearing) in the FL, considerably lower than the Lyt 2^+ cells. This proportional difference is not unusual since the θ marker (Thy 1) can be independent of the cell types showing the Lyt markers (Ledbetter *et al.*, 1980). Therefore, it is tempting to speculate that the fluctuations observed with regard to the Lyt and θ markers, particularly in the FL, reflect variations in migration processes. However, in this regard, other explanations are possible, e.g., cells expressing θ in the FL may die off and be replaced by new cells showing this marker. Therefore, there is a need to address, in BP-exposed progeny, in greater detail during fetogenesis, extrathymic T cell differentiation, since the normal pattern of T cell distribution is decidely disrupted.

In the ontogenesis of thymic cells from 10 to 17 days gestation, the FL provides precursor (stem) cells that lead to the maturation of T cells (Stutman,

1978; Pyke and Bach, 1979). The nature of the Lyt positive cells in the FL needs to be characterized. From the data reported here we cannot rule out that precursors of T cells are not supplied by the FL. It is possible that some of these cells die (toxic effect of BP?) before reaching the thymus or in the thymus, leading to a depletion of the subsets, rather than there being a maturational change in this organ.

The quantitative changes in T cells and subsets in the thymus and their disproportionate accumulation in the FL and spleen may have important consequences in the contribution of T cell maturation events for leading to emerging patterns of immunologic responsiveness (Murgita and Wigzell, 1981). Characterization of the precise functional profile of the cells quantified in the BP-exposed progeny will allow us to establish their identity and provide an approach to evaluate changes that may be occurring in the differentiative process. From our current data (see also Discussion, pg. 12), further identification of changes in the functional capacity of cellular entities involved would strongly suggest that BP evokes uncommon differentiative pathways, i.e., the appearance of new immunologic markers (also suggested in adults by Wojdani *et al.*, 1983) representing distinct functions. The persistence of these or patterns emerging from them could lead to subtle abnormalities which may be directly correlated with increased susceptibility for tumorigenesis.

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References

Aggarwal AL, Raiyani CV, Patel PD, Shah PG, Chatterjee SK (1982) Assessment of exposure to benzo(a)pyrene in air for various population groups in Ahmedabad. *Atmos. Environ. 16:867.*

Alexandrov VA (1983) Role of the maternal organism in transplacental carcinogenesis. IARC *Sci. Publ. 51:65.*

Alfred LJ, Wojdani A, Nieto M, Perez R, Yoshida G (1983) A chemical carcinogen, 3-methylcholanthrene, alters T-cell function and induces T-suppressor cells in a mouse model system. *Immunology 50:207.*

Baldwin RW, Robins RA (1977) Induction of tumor immune responses and their interaction with the developing tumor. *Contemp. Top. Mol. Immunol. 6:177.*

Bulay DM, Wattenberg LW (1971) Carcinogenic effects of polycyclic hydrocarbon carcinogen administration to mice during pregnancy on the progeny. *J. Natl. Cancer Inst. 46:397.*

Cantor H, Boyse EA (1975) Functional subclasses of T lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. *J. Exp. Med. 141:1376.*

Ceredig R, Sekaly RP, MacDonald HR (1983) Differentiation *in vitro* of Lyt 2^+ thymocytes from embryonic Lyt 2^- precursors. *Nature 303:248.*

Clark EA, Harmon RC (1980) Genetic control of natural cytotoxicity and hybrid resistance. *Adv. Cancer Res. 31:227.*

Dean JH, Luster MI, Boorman GA, Lauer LD, Luebke RW (1983) Selective immunosuppression resulting from exposure to the carcinogenic congener of benzopyrene in B6C3F1 mice. *Clin. Exp. Immunol. 52:199.*

Griciute L (1979) Carcinogenicity of polycyclic aromatic hydrocarbons. In: *Environmental Carcinogens, Selected Methods of Analysis*, Vol. 3. Ed. H. Egany, Lyon, France, IARC, pp 3-15.

Grimmer G, Pott E (1983) Occurrence of PAH. In: *Environmental Carcinogens: Polycyclic Aromatic Hydrocarbons*. Ed. G. Grimmer, Boca Raton, Florida: CRC Press Inc., pp. 61-128.

Head JR, Billingham RE (1982) Immunobiological aspects of the maternalfetoplacental relationship. In: *Clinical Aspects of Immunology*, 4th ed. Vol. 1, Eds P.J. Lachman and D.K. Peters, Blackwell Scientific Publications, Oxford, pp. 243-282.

Huhti E (1981) Smoking and lung cancer-now. *Eur. J. Resp. Dis. 62: 147.*

Jones SL, Johnson RA, Urso P (1986) Observations on B cells of progeny and mothers exposed to benzo(a)pyrene at midgestation. *Fed. Proc. 45: 258.*

Julius MH, Simpson E, Herzenberg LA (1973) A rapid method for isolation of functional thymus-derived lymphocytes. *Eur. J. Immunol. 3:645.*

Ledbetter JA, Rouse RN, Micklem HS, Herzenberg LA (1980) T cell subsets defined by expression of Lyt-1, 2, 3 and Thy-1 antigens. Two parameter immunofluorescence and cytotoxicity analysis with monoclonal antibodies modifies current views. *J. Exp. Med. 152:280.*

Murgita RA, Wigzell H (1981) Regulation of immune functions in the fetus and newborn. *Prog. Allergy 29:54.*

Nikinova TV (1977) Placental action of benzo(a)pyrene and pyrene. *Bull. Exp. Biol. Med. 84:1025.*

Perry LL, Greene MI (1981) T cell subset interactions in the regulation of syngeneic tumor immunity. *Fed. Proc. 40:39.*

Pyke KW, Bach J-F (1979) The *in vitro* migration of murine fetal liver cells to thymic rudiments. *Eur. J. Immunol. 9:317.*

Scheid MP, Goldstein G, Hammerling U, Boyse EA (1975) Lymphocyte differentiation from precursor cells *in vitro*. *Ann. N. Y. Acad. Sci. 249:531.*

Shendrikova A, Aleksandrov VA (1974) Comparative penetration of polycyclic hydrocarbons through the rat placenta into the fetus. *Bull. Exp. Biol. Med. 77:169.*

Skov CB, Holland JM, Perkins EH (1976) Development of fewer tumor colonies in lungs of athymic nude mice after intravenous injection of tumor cells. *J. Natl. Cancer Inst. 56:193.*

Sokal RR, Rohlf FJ (1981) *Biometry, 2nd ed,* W.H. Freeman, San Francisco.

Stjernsward J (1966) Age-dependent tumor-host barrier and effect of carcinogen induced immunodepression on rejection of isografted methylcholanthrene-induced sarcoma cells. *J. Natl. Cancer Inst. 37:505.*

Stjernsward J (1969) Immunosuppression by carcinogens. *Antibiot. Chemother. 15:213.*

Stutman O (1978) Intrathymic and extrathymic T cell maturation. *Imm. Rev. 42:138.*

Tomatis L, Parmi L (1971) Effect of Perinatal administration of 7, 12-dimethylbenz(a)anthracene on the later response to subcutaneous Teflon implant. *Tumori 57:55.*

Urso P, Gengozian N (1973) T cell deficiency in mouse allogeneic radiation chimeras. *J. Immunol. 111:712.*

Urso P, Gengozian N (1980) Depressed humoral immunity and increased tumor incidence in mice following *in utero* exposure to benzo(a)pyrene. *J. Toxicol.*

Environ. Health 6:569.

Urso P, Gengozian N (1980a) Suppression of humoral and cell-mediated immune response after *in utero* exposure to benzo(a)pyrene. *Fed. Proc. 39:775.*

Urso P, Gengozian N (1982) Alterations in the humoral immune response and tumor frequencies in mice exposed to benzo(a)pyrene and X-rays before or after birth. *J. Toxicol. Environ. Health 10:817.*

Urso P, Gengozian N, Rossi RM (1983) Modulation of Immune Capacity after insult with benzo(a)pyrene (BP) and radiation during fetal life. *Fed. Proc. 42:1298.*

Urso P, Gengozian N (1984) Subnormal expression of cell-mediated and humoral immune responses in progeny disposed toward a high incidence of tumors after *in utero* exposure to benzo(a)pyrene. *J. Toxicol. Environ. Health 14:559.*

Urso P, Johnson RA (1987) Suppression by Lyt 1^+ and Lyt 2^+ fetal liver cells emerging after *in utero* insult with carcinogenic benzo(a)pyrene may be mediated by Lyt 1^+ cells. *Fed. Proc. (In Press).*

Vesselinovitch SD, Kyriazis AP, Mikailovich N, Rao KVN (1975) Conditions modifying development of tumors in mice at various sites by benzo(a)pyrene. *Cancer Res. 36:2948.*

Welch RM, Gommi B, Alvares AP, Conney AH (1972) Effect of enzyme induction on the metabolism of benzo(a)pyrene and 3'-methyl-4-monomethylaminoazobenzene in the pregnant and fetal rat. *Cancer Res. 32:973.*

Winn HJ (1961) Immune Mechanisms in homotransplantation. II. Quantitative assay of the immunologic activity of lymphoid cells stimulated by tumor homografts. *J. Immunol. 86:228.*

Wojdani A, Nieto M, Alfred LJ (1983) Carcinogenic PAH compounds suppress cell mediated immune functions, and alter T-cell subsets in splenic lymphocyte populations. *Proc. Am. Assoc. Cancer Res. 24:242.*

Wojdani A, Alfred LJ (1984) Alterations in cell-mediated immune functions induced in mouse splenic lymphocytes by polycyclic aromatic hydrocarbons. *Cancer Res. 44:942.*

Wunderlich V, Tetzlaff I (1973) Applikation van ^{3}H-benz-(*a*)-pyren an gravide mause und ratten: Inkorporaton der radioaktivitat in subcellulare fraktionen verschiedener gewebe. *Archiv fur Geschulstforschung 42:95.*

FIGURE LEGENDS

Fig. 1. Changes in body weight and weight of lymphoid organs of progeny exposed to corn oil or unexposed controls (○), or BP experimentals (●) at midgestation. N=neonate. Vertical lines represent standard deviations (SD) from the mean. Significant difference from the means for experimentals and controls at each time interval was determined by a one-way analysis of variance (p values given in the text). Student's t-test gave similar p values. Fifteen to 25 determinations per point; 3 to 5 litters.

Fig. 2. Changes in cell numbers in lymphoid organs of control (○) and experimental (●) progeny after exposure at midgesation. N=neonate. SD (vertical lines), statistical analyses and number of determinations per point as in Fig. 1.

Fig. 3. Frequency changes in T (θ^+)-cells and subsets in control (□) or experimental (■) progeny exposed at midgestation. (See p. 6 of text for calculation of percent cells). SD (vertical lines), statistical analyses and number of determinations per point as in Fig. 1.

Fig. 4. Changes in absolute numbers of T cells and subsets in lymphoid organs of control (○) or experimental (●) progeny exposed during midgestation. N=neonate. (See p. 9 for calculation of T cell numbers). SD (vertical lines), statistical analyses and number of determinations per point as in Fig. 1.

Fig. 5. Proportional changes in T cell subsets in control (□) or experimental (▨) progeny aftger birth following *in utero* exposure. Five determinations per bar. SD and statistical analyses as in Fig. 1.

Fig. 6. Growth of tumor following sc injection of syngeneic normal recipients with tumor cells (10^5) only (▲), tumor cells mixed with $5x10^6$ spleen cells

from controls (○) and tumor cells mixed with $5x10^6$ spleen cells from experimentals (●). SD (vertical lines) as in Fig. 1. Significant diferences between means at each time interval determined by a two-way analysis of variance and Student's t-test; p values for each interval represent differences between experimentals and controls at that interval. Eighteen to 23 determinations per point involving 5 to 7 experimental (BP-exposed) or control (normal or corn oil exposed) donors (i.e., 5 to 7 experiments).

Fig. 7. Formation of tumor colonies in the lungs of control (□) or experimental (▨) progeny receiving 10^5 tumor cells intravenously 15 to 21 days earlier. Vertical lines are SD. Statistical analysis as in Fig. 1. Numbers in parentheses represent number of recipients.

TABLE I

Relative proportions of Lyt 1^+ and Lyt 2^+ cells in lymphoid tissues of progeny following exposure to BP at 11 to 13 days of gestation

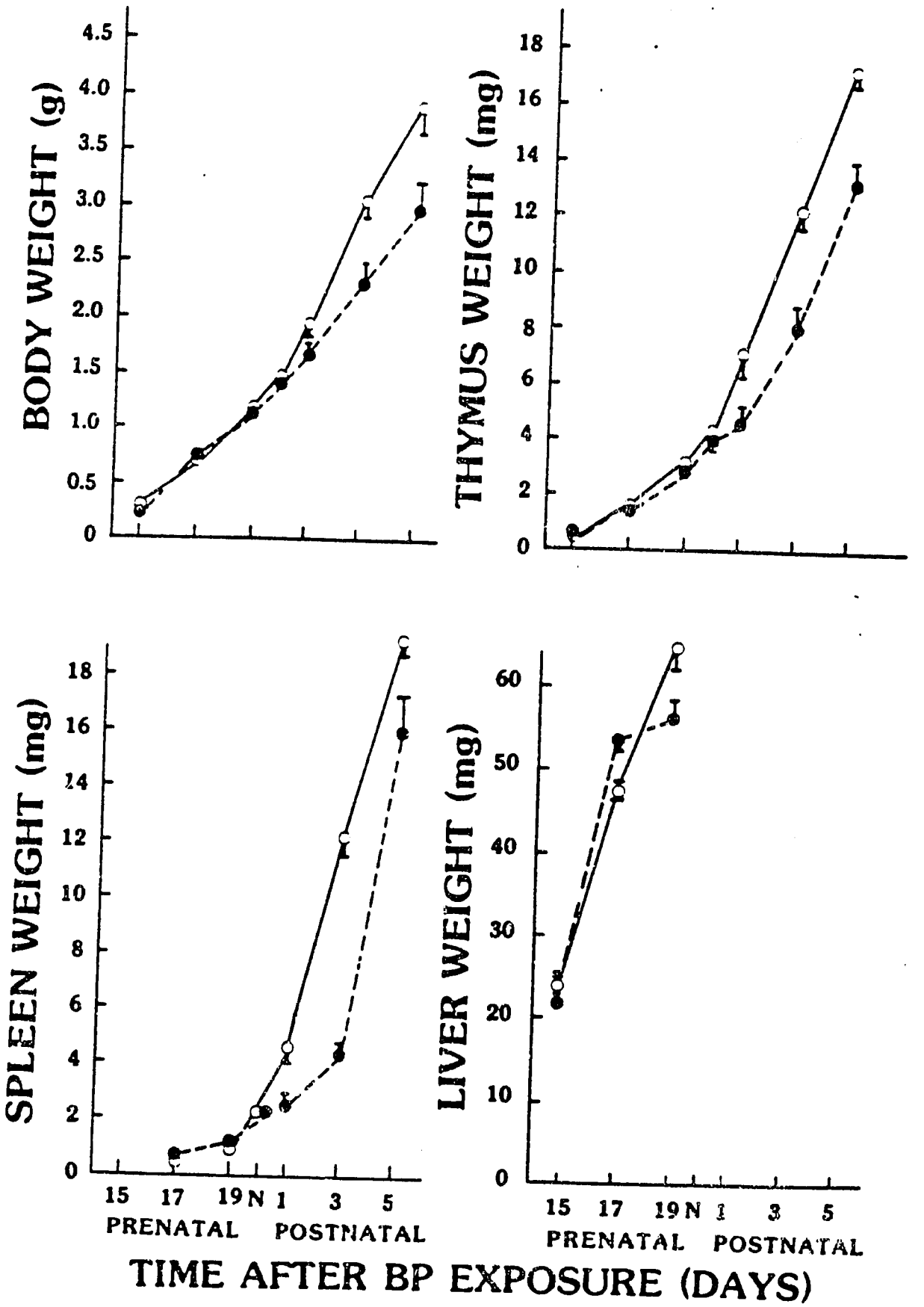
Treatment and Organ	Number of Lyt 1^+ cells/number of Lyt 2^+ cells ± SD on:			
	Prenatal Day 17	Prenatal Day 19	Postnatal Day 1	Postnatal Day 3
CO thymus	1.11±0.10	1.60±0.12	1.61±0.10	1.22±0.04
BP thymus	1.12±0.12	1.00±0.10	1.12±0.07	1.30±0.05
CO spleen	1.34±0.10*	1.50±0.08*	2.75±0.64*	1.24±0.34*
BP spleen	0.62±0.03	0.59±0.12	0.75±0.08	0.40±0.11
CO liver	1.94±0.12*	1.13±0.22*		
BP liver	0.31±0.10	0.22±0.04		

SD = STANDARD DEVIATION

CO = Exposure to corn oil (control); BP = Exposure to BP (experimental)

* = Significant at $p < 0.01$ to < 0.0001.

FIG. 1


BODY WEIGHT (g)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
THYMUS WEIGHT (mg)
18
16
14
12
10
8
6
4
2
0
SPLEEN WEIGHT (mg)
18
16
14
12
10
8
6
4
2
0
LIVER WEIGHT (mg)
60
50
40
30
20
10
0
15 17 19 N 1 3 5
PRENATAL POSTNATAL
15 17 19 N 1 3 5
PRENATAL POSTNATAL
TIME AFTER BP EXPOSURE (DAYS)

22

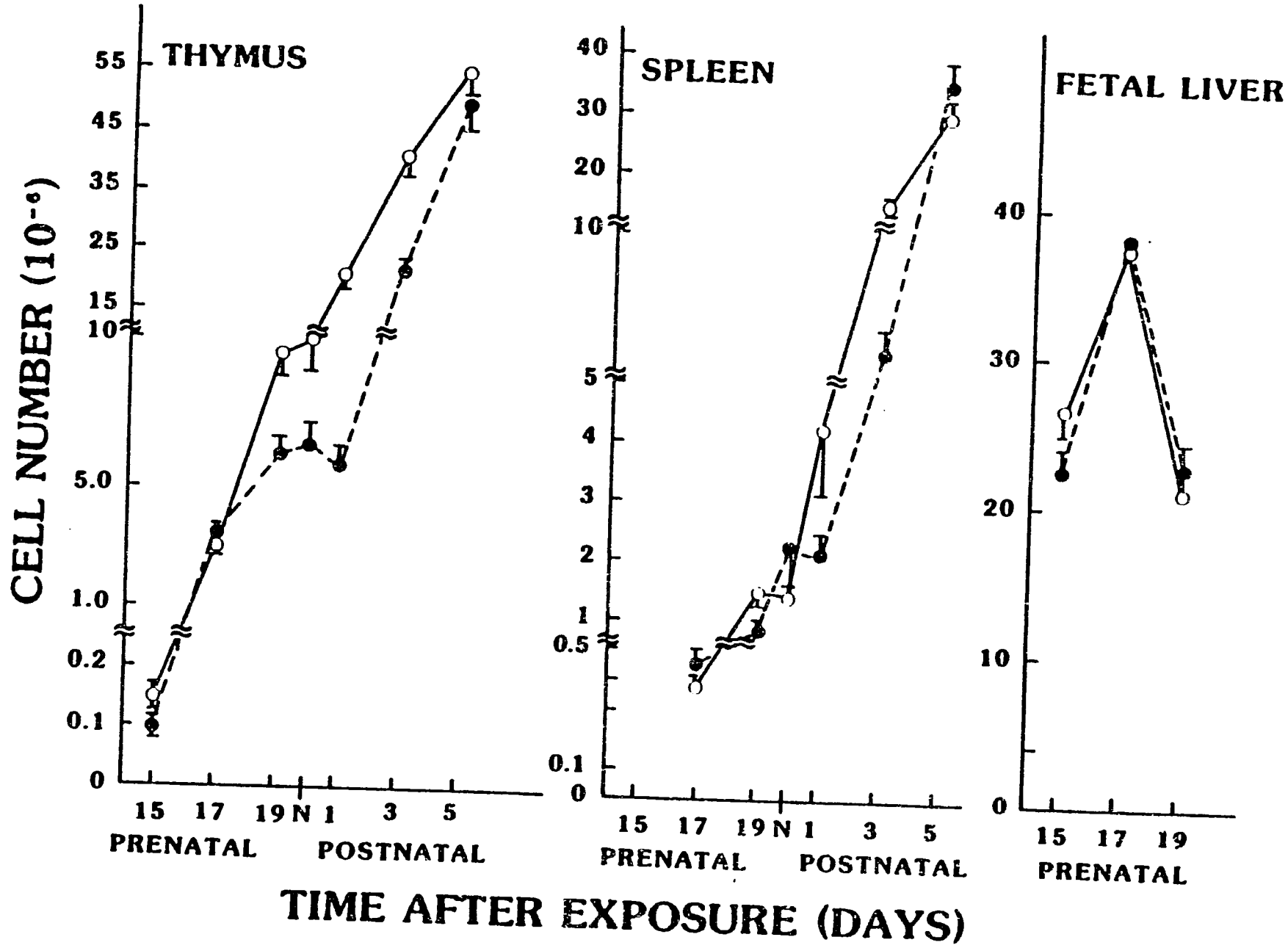

THYMUS
SPLEEN
FETAL LIVER
CELL NUMBER (10^{-6})
55
45
35
25
15
10
5.0
1.0
0.2
0.1
0
40
30
20
10
5
4
3
2
1
0.5
0.1
0
40
30
20
10
0
15 17 19 N 1 3 5
PRENATAL POSTNATAL
15 17 19 N 1 3 5
PRENATAL POSTNATAL
15 17 19
PRENATAL
TIME AFTER EXPOSURE (DAYS)

Fig. 2

FIG. 3

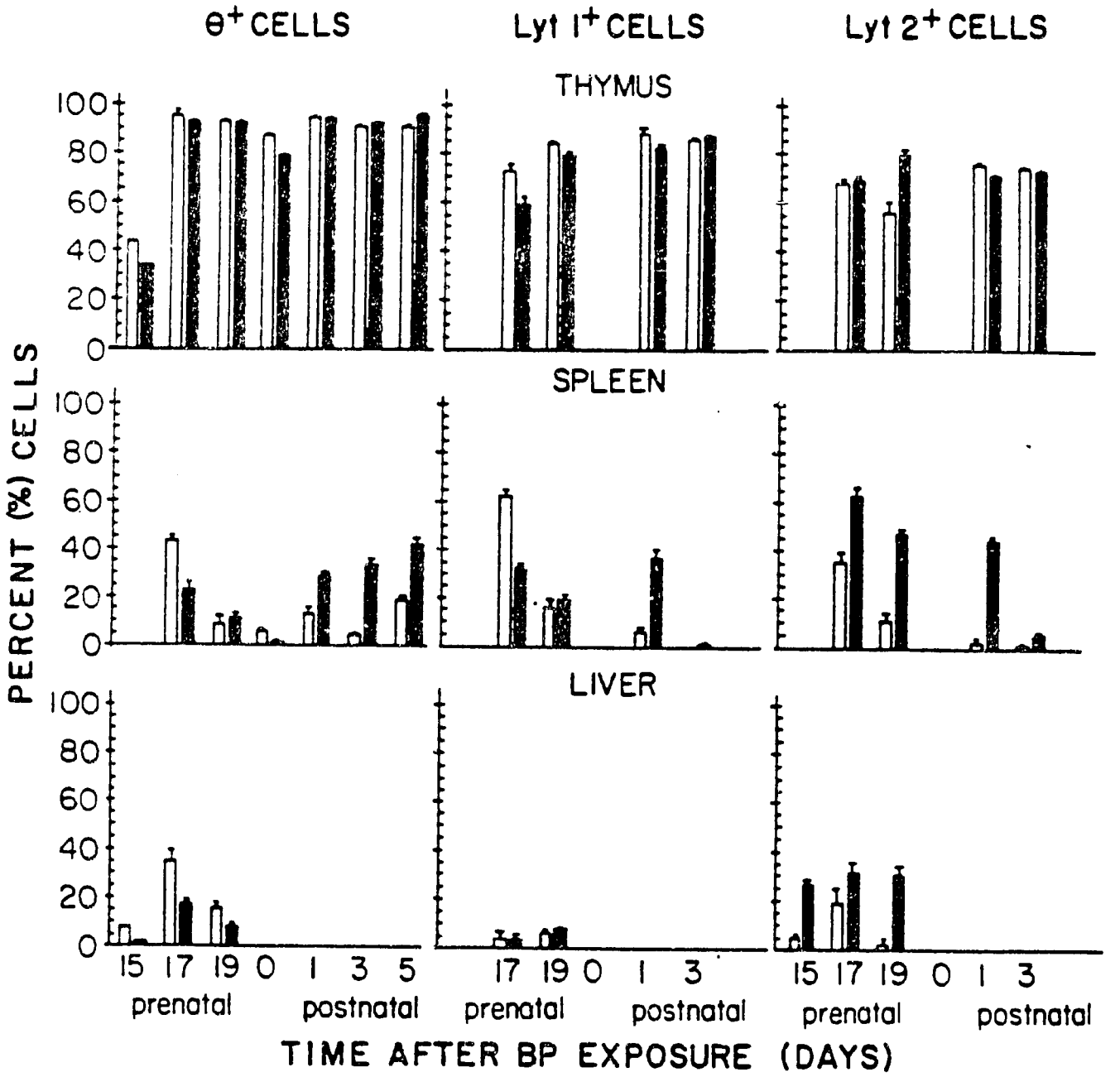
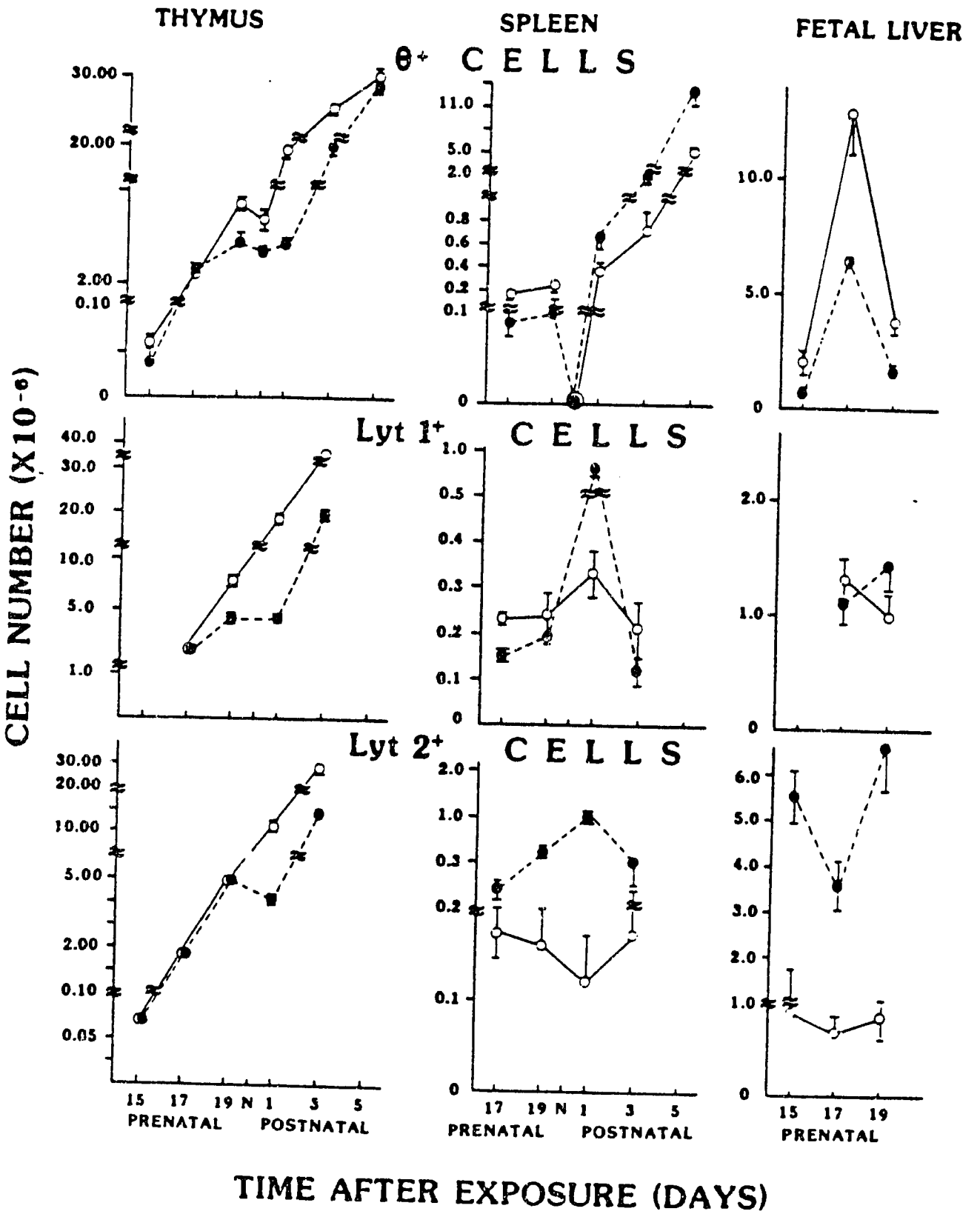

θ+ CELLS
Lyt 1+ CELLS
Lyt 2+ CELLS
THYMUS
SPLEEN
LIVER
PERCENT (%) CELLS
100
80
60
40
20
0
15 17 19 0 1 3 5
prenatal
postnatal
17 19 0 1 3
prenatal
postnatal
15 17 19 0 1 3
prenatal
postnatal
TIME AFTER BP EXPOSURE (DAYS)

FIG. 4


THYMUS
SPLEEN
FETAL LIVER
θ+ CELLS
Lyt 1+ CELLS
Lyt 2+ CELLS
CELL NUMBER (X10-6)
30.00
20.00
2.00
0.10
0
11.0
5.0
2.0
0.8
0.6
0.4
0.2
0.1
10.0
5.0
40.0
30.0
20.0
10.0
5.0
1.0
1.0
0.5
0.4
0.3
0.2
0.1
2.0
1.0
30.00
20.00
10.00
5.00
2.00
0.10
0.05
2.0
1.0
0.3
0.2
0.1
6.0
5.0
4.0
3.0
2.0
1.0
15 17 19 N 1 3 5
PRENATAL POSTNATAL
17 19 N 1 3 5
PRENATAL POSTNATAL
15 17 19
PRENATAL
TIME AFTER EXPOSURE (DAYS)

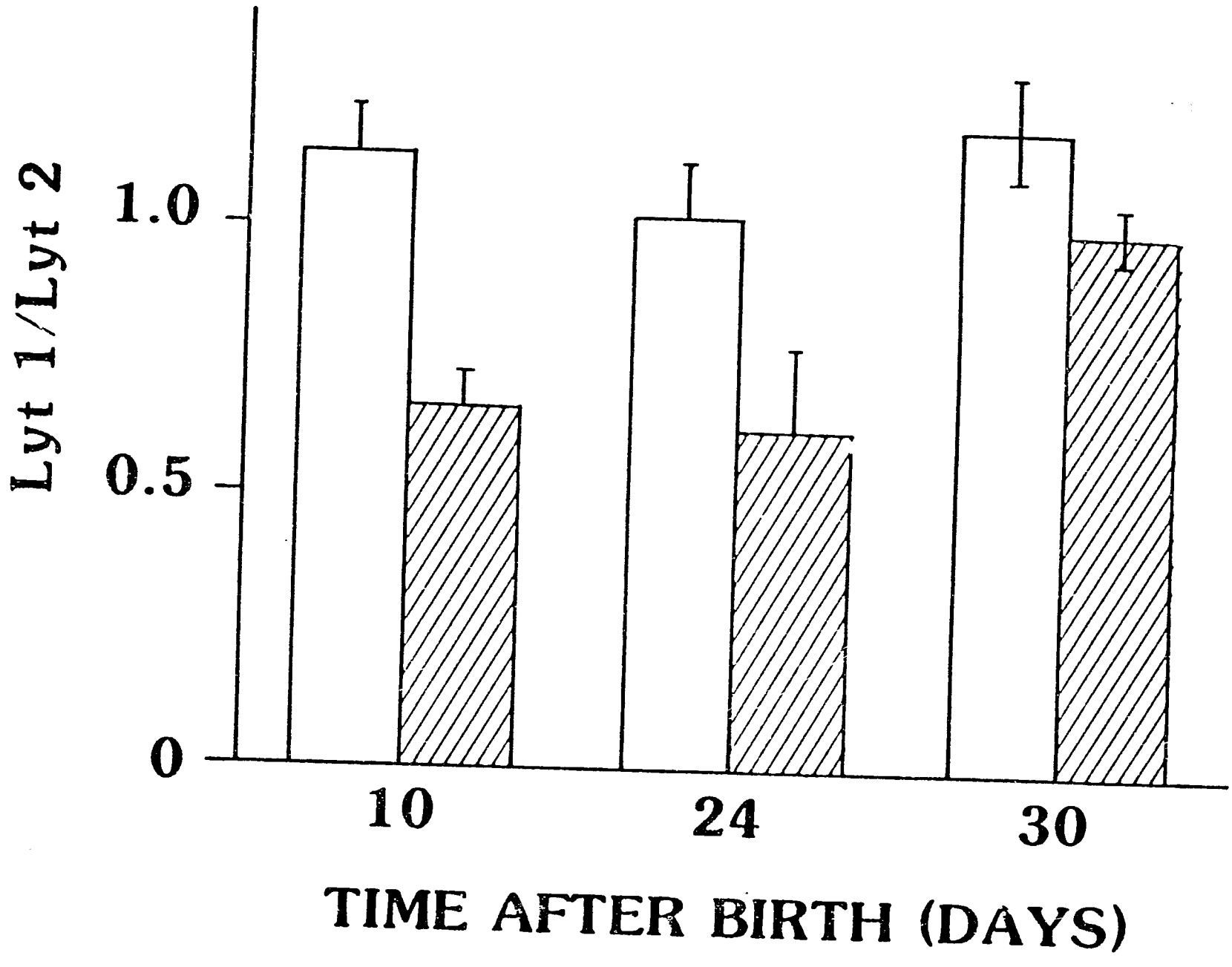


FIG. 5

FIG. 6

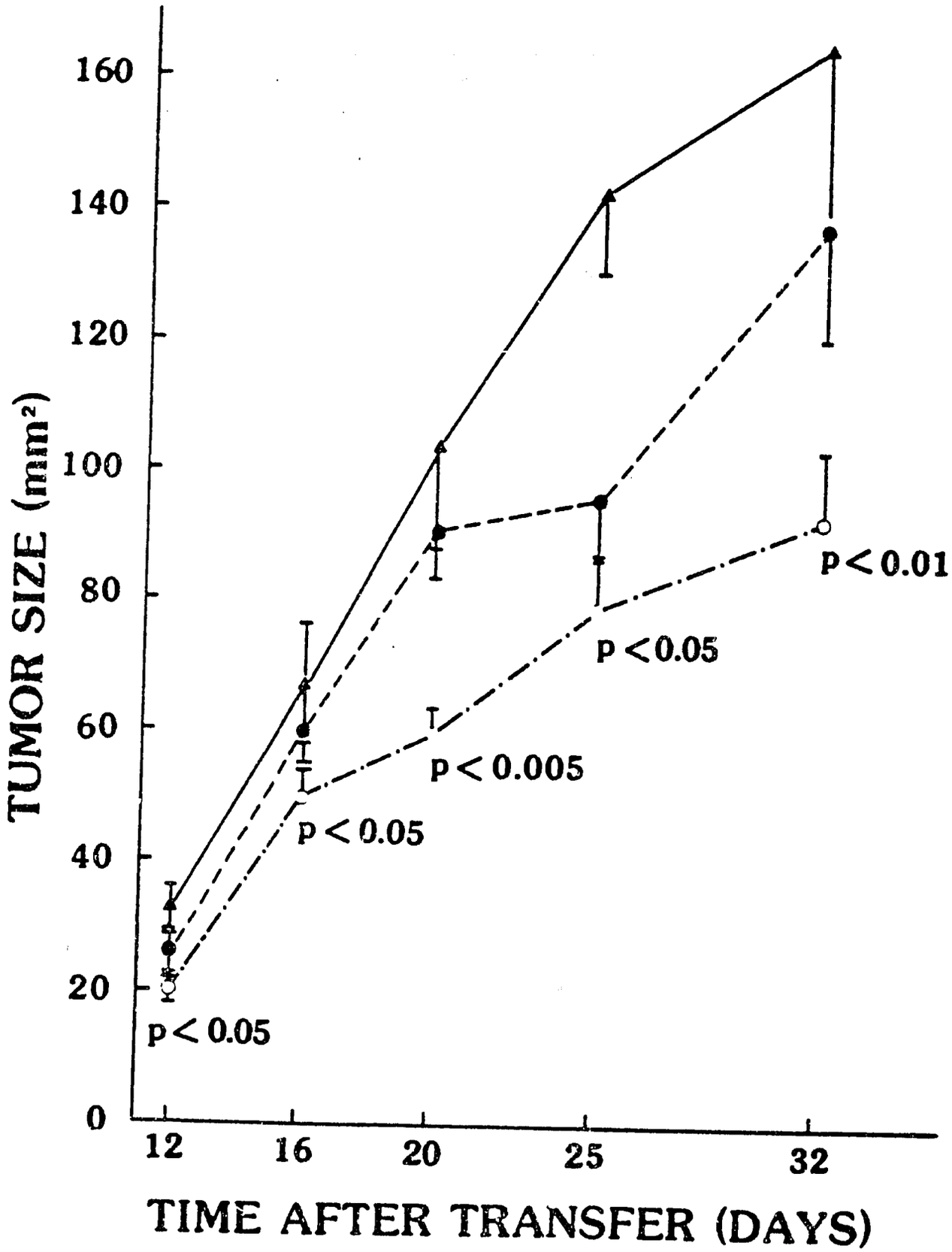
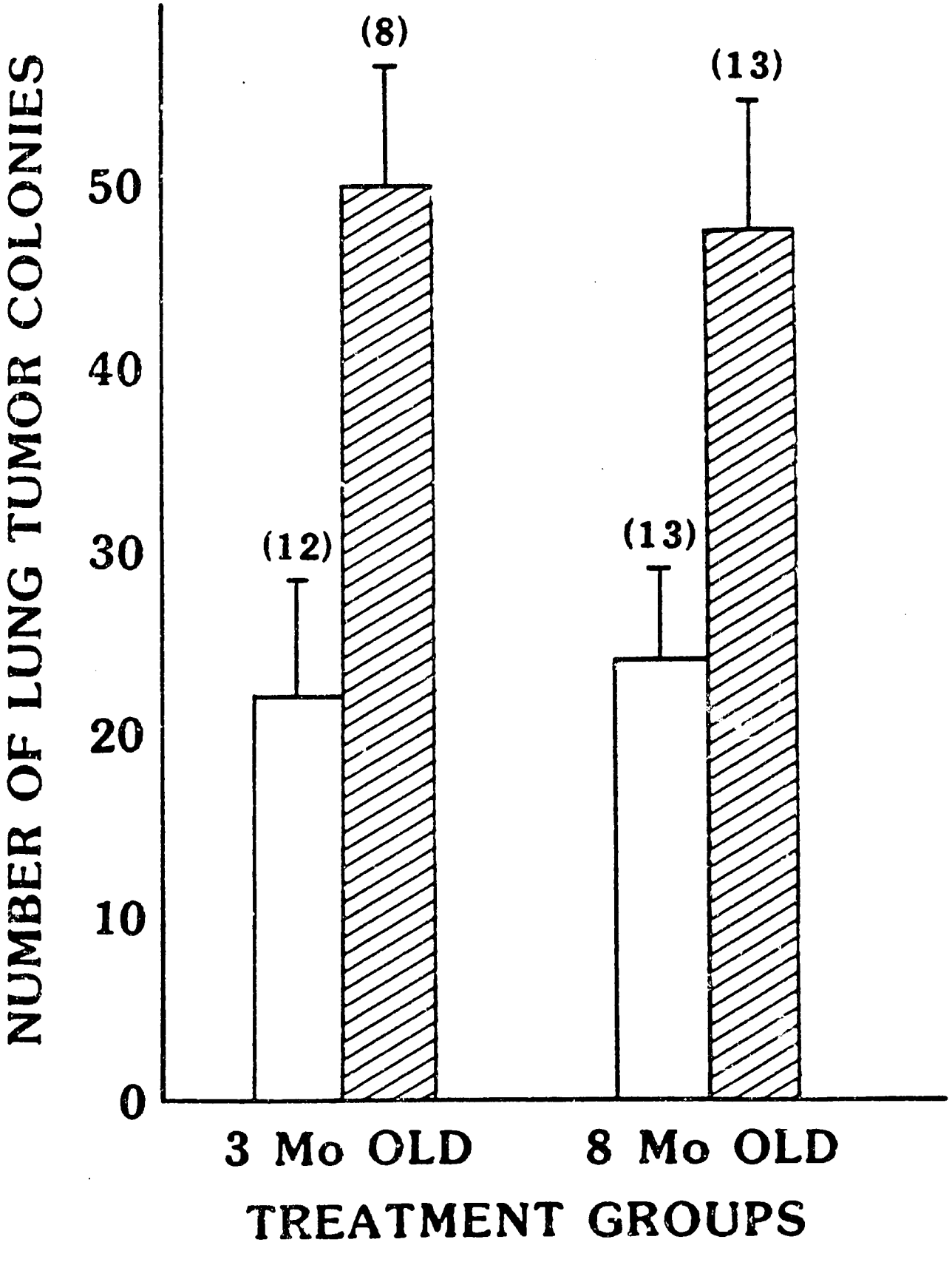

160
140
120
100
80
60
40
20
0
TUMOR SIZE (mm²)
12
16
20
25
32
TIME AFTER TRANSFER (DAYS)
P < 0.05
P < 0.05
P < 0.005
P < 0.05
P < 0.01

FIG. 7


NUMBER OF LUNG TUMOR COLONIES
0
10
20
30
40
50
(12)
(8)
(13)
(13)
3 Mo OLD
8 Mo OLD
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