RESPONSE OF TUMOUR-RELATED AND NORMAL LYMPHOCYTES TO ANTIGENS ON FIBROBLASTS FROM EMBRYOS OF VARYING AGE*

R. M. GORCZYNISKI

From the Ontario Cancer Institute and Department of Medical Biophysics, University of Toronto, 500 Sherbourne Street, Toronto, Ontario, Canada M4X 1K9

Received 5 September 1977  Accepted 13 January 1978

Summary.—The in vitro cytotoxic immune response of spleen lymphocytes from primiparous and tumour-related mice to embryonic cells from embryos of varying age and tumour cells has been investigated. The results indicate that lymphocytes from both primiparous and tumour-related (i.e., tumour-bearing or tumour-excised) animals give a response which is greater than that from cells from control mice (“virgin cells”). Moreover, in this putative anamnestic response the immune cells detect antigenic differences in the cell populations of embryos of varying age, which are not as readily demonstrable when cytotoxicity is derived from virgin cells. As a further indication of the in vivo priming to embryo-associated antigens, the data show that the precursors of cytotoxic cells apparently undergo a blastogenic response in the presence of embryo antigen, and revert to small quiescent cells when antigen is removed, in a way entirely analogous to that described for reactivity of mixed leucocyte cultures to antigens of the major histocompatibility complex. Finally, it seems that in animals immediately after removal of embryonic antigen (and to a lesser degree in virgin or late-embryo-immune mice) there exists a suppressor cell population which inhibits an anti-embryo cytotoxic response far more than an anti-allograft response.

The immune response of adult mice challenged with histoincompatible cells results in the generation of antigen-specific cytotoxic T lymphocytes (CTL) able to recognize the H-2K/D specificities expressed by the antigen stimulating cells (Alter et al., 1973). Furthermore, such alloantigen-primed CTL have been shown to confer, in a similar antigen-specific fashion, protection against allogeneic tumour-cell growth in vivo (Cohen, Globerson and Feldman, 1971).

In contrast to these findings, adult cells stimulated with embryonic antigens (expressed on naturally occurring or chemically/virally induced tumours) have not been reported to show specificity towards embryonic antigens, even on tumours of a given histological type (Baldwin, Glaves and Vose, 1972). Indeed cross-reactivity has been reported for embryo antigens from animals which differed at the major histocompatibility complex (Gorcynski, 1976a). A number of groups have reported that immunization with foetal tissue is ineffective as a means of inducing tumour-immunity (Pearson and Freeman, 1968; Ting, 1968; Basombrio and Prehn, 1972). In contrast to this, several workers, using SV40-induced tumours (Coggin, Ambrose and Anderson, 1970) and Rauscher-leukaemia-virus-induced tumours (Hanna, Tennant and Coggin, 1971) have suggested that foetal immunization can confer tumour immunity. Perhaps even more striking are the examples where foetal immunization has been shown to enhance the growth of tumours expressing embryo-associated antigens (Castro et al., 1973; Parmiani and Lembo, 1974). In addition, a recent communication from this laboratory showed that

* The author regrets that no reprints of this article will be available.
while lymphocytes from normal healthy adults spontaneously developed cytotoxicity directed against embryonic antigen determinants in tissue culture, the reactive cells were not universally CTL (Gorzynski, 1976b). In particular, in female lymphocytes, the reactivity seemed to be associated with a B lymphocyte/macrophage cytotoxic response.

In an effort to examine in a more systematic fashion the potential reasons for the differences cited above, the ability of lymphocytes taken from animals at various stages of pregnancy or tumour growth to show reactivity to embryoid fibroblasts from embryos of varying ages has been examined. The data described below indicate that lymphocytes from animals pre-exposed to embryonic antigens show a fine specificity not shown by virgin cells in terms of in vitro induction of secondary cytotoxicity. Moreover, evidence is presented to show that during tumour (or embryo) growth and at early times after tumour (or embryo) excision a cell population exists which is capable of inhibiting the immune response to embryonic antigens (and, considerably less effectively, to histocompatible cells).

MATERIALS AND METHODS

Mice.—C3H/HeJ and (C3H × C57BL/6J)F1 mice were obtained from the Jackson Laboratories, Bar Harbour, Maine. All animals were kept 5 to a cage and given food and water ad libitum.

Termed pregnancies were induced by putting male and female mice together for 16–20 h. Pregnant mice (vaginal-plug technique) were separated from the remaining mice. Birth occurred at 19 to 20 days of gestation.

Tumours.—Retired breeder mice from Jackson Laboratories were inspected twice weekly for the appearance of spontaneous tumours. When such a tumour was ~1 cm³ in volume, the animals were killed by cervical dislocation and the tumour removed aseptically in phosphate-buffered saline (PBS). A sample of the tumour was taken to the Histology/Pathology Department for analysis. Unless otherwise specified, all tumours used throughout these experiments were adenocarcinomas of female mice. Neoplasms were disaggregated with a mixture of trypsin (2.5% solution, Grand Island Biological Company, Grand Island, New York), crude collagenase (CLS 11, 140 u/mg; Worthington Biochemical Corporation, Freehold, New Jersey) and deoxyribonuclease (DNase 1, B Grade, 7 × 10⁴ Dornase units/mg; Calbiochem, San Diego, California) as described by Russell et al. (1976). All enzymes were used at a concentration of 0.1 mg/ml and the tumour cells were harvested from the digestion flask at 30 min intervals for a total of 90 min. The mean yield per tumour was 1–4 × 10⁷ cells.

2.5 × 10⁵ tumour cells were transferred s.c. into the back (in 0.2 ml PBS) to each of 15 8-week-old female C3H/HeJ mice, and the growth of these tumours followed. In the case of these primary recipient animals, when the tumour was about 1 cm³ the animals were either killed and used as a source of test lymphocytes, or anaesthetized with Nembutal and the tumour mass excised.

Fibroblasts.—Embryo fibroblast cells were prepared from C3H embryos of varying ages as described before (Gorzynski, 1976a). Fibroblasts, primary tumour cells, and fresh single cell suspensions of embryo cells (prepared as for the tumour suspension above) were stored in liquid N₂ at a concentration of 2 × 10⁶ cells/ml in αF₃₀ (α-MEM supplemented with 30% FCS) with 15% dimethyl sulphoxide. All cell counts here and subsequently refer to viable cells as determined by trypan-blue-dye exclusion.

Cell preparation, irradiation, ³¹Cr-cytotoxicity assays and velocity sedimentation.—These have been described in detail in previous publications (Gorzynski, 1976a, c; Miller and Phillips, 1969).

Microcytotoxicity assay.—The assay was a modification of that previously described (Gorzynski, 1976a). Target cells for the microcytotoxicity assay were ³H-proline labelled (630 mCi/mmol, Radiochemicals Centre, Amersham, England) embryonic fibroblasts. ³H-proline was used, rather than ³H-thymidine as previously, in view of the superior uptake of label, lower rate of spontaneous release, and decreased re-utilization of the label by lymphocytes or unlabelled embryonic fibroblasts in proline containing αF₁₀ (Bean, Rosen and Oettgen, 1973).
After labelling the embryonic fibroblasts with 150 μCi of 3H-proline for 18 h in 3 ml of proline-free αF5 the fibroblasts were washed twice in PBS, trypsinized and washed twice in αF10. The cells were then resuspended to a concentration of 10^4/ml and 100 μl of cells dispersed into each well of a 96-well (6 mm) Limbro microtest plate (Limbro Chemical Company, New Haven, Connecticut). After allowing 3 h for the fibroblasts to adhere to the bottom of the wells, effector cells were added in 100 μl to each well. Various concentrations of effector cells (always in 100 μl) were used, and control groups contained either αF10 alone (medium control) or detergent (total releaseable ct/min). All groups were set up in triplicate.

After 24 h the plates were centrifuged (500 g for 5 min at 4°C) and 100 μl of the supernatant in each well harvested, dissolved in 5 ml Aquasol (New England Nuclear, Boston, Mass.) and counted in a scintillation counter. Percent specific cytotoxicity was calculated as:

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100 \times \left( \frac{(\text{ct/min experimental} - \text{ct/min spontaneous})}{(\text{ct/min detergent} - \text{ct/min spontaneous})} \right)
\]

Where possible, all data are expressed as arithmetic mean ± s.e. mean. Within any given experiment described herein, the target cells were from the same frozen stock.

Cell cultures.—All cells sensitized against allogeneic or syngeneic cells were cultured in 16 mm Limbro culture plates, in a total volume of 2-5 ml αF10 per well. The plates were initially pre-cultured for 4 h at 37°C in an humidified CO₂-containing atmosphere with 2·0×10⁵ normal peritoneal macrophages per well (in 0·5 ml αF10). Then 5×10⁴ freshly prepared embryo cells (not embryo fibroblasts) or tumour cells were added to each well in 200 μl αF10, and the plates were returned to the incubator for a further 2 h. The individual wells were then washed twice, each time with 2·0 ml of pre-warmed αF10, the plates irradiated with 2500 rad in a 137Cs irradiator, and lymphocytes added to the wells described. The plates were now returned to the incubator for 5 days, after which the cells in the wells were harvested, washed twice in αF10 and assayed as described.

RESULTS

Cytotoxicity of freshly prepared lymphocyte populations to embryonic fibroblasts made from embryos of varying ages.

Earlier reports from this and other laboratories (Gorczynski, 1976a; Baldwin et al., 1974; Hellström and Hellström, 1976) have shown that lymphocytes from tumour-bearing (and tumour-excised) mice and primiparous mice are naturally cytotoxic for embryonic fibroblasts prepared from mid-gestation embryos. In addition, it has proved possible to sensitize allegedly virgin cells to embryo-associated antigens under tissue-culture conditions, though no extensive analysis of such sensitization has been performed with cells already primed in vivo. In order to investigate whether in vivo priming during pregnancy or tumour growth takes place to unique antigens associated with cells from embryos of varying ages, we have therefore compared the cytotoxicity of such sensitized lymphocytes during the ongoing primary and secondary response.

The data of Table I (the pooled results from 5 independent experiments) show the cytotoxicity of spleen lymphocytes, prepared from animals at different stages of in vivo sensitization to embryonic antigen, to embryo fibroblasts prepared from different ages of embryo. As already reported, freshly prepared virgin lymphocytes showed negligible cytotoxicity to the fibroblast targets, and the same was true for lymphocytes prepared from animals at long times after tumour resection. In contrast, during pregnancy or tumour growth, cytotoxicity to embryonic fibroblasts was apparent, with a degree of specificity attached to the cytotoxicity. Thus lymphocytes early in pregnancy showed optimum cytotoxicity to fibroblasts from young embryos, and only later did cytotoxicity to fibroblasts from older embryos appear.

Specificity of re-stimulation of embryo-primed cells in tissue culture

The data in Table I are consistent with the notion that different embryonic anti-
Table I.—Cytotoxicity of Fresh Spleen-Cell Preparations for Fibroblasts from Embryos of Different Ages

| Source of spleen cells† | Cef₈ | Cef₁₁ | Cef₁₄ | Cef₁₈ |
|------------------------|------|-------|-------|-------|
| Normal adult           | 1·0±0·2 | 0·8±0·1 | 1·1±0·3 | 1·1±0·3 |
| Pregnant (8 days)      | 1·2±0·3 | 1·0±0·2 | 0·9±0·4 | 1·1±0·3 |
| Pregnant (11 days)     | 4·1±1·9* | 4·7±2·1* | 4·2±2·1* | 0·9±0·2 |
| Pregnant (14 days)     | 2·7±0·4* | 8·9±2·8* | 8·7±3·6 | 1·6±0·4 |
| Pregnant (18 days)     | 6·8±3·1* | 8·6±2·4* | 10*     | 3·2±1·1* |
| 20 days post-partum    | 2·4±0·9* | 2·1±0·6 | 4·1±0·9* | 1·1±0·3 |
| Tumour bearer          | 3·4±0·9* | 4·7±1·5* | 4·8±2·2* | 1·1±0·3 |
| 10 days after tumour resection (tumour-free) | 4·8±1·6* | 6·1±2·9* | 4·7±1·6* | 1·6±0·9 |
| 30 day tumour resection (tumour-free) | 2·2±1·1 | 1·9±0·4 | 2·5±1·1 | 1·1±0·4 |

* P<0·05.
† Spleen cells were harvested from individual mice of the type shown. Tumour cells were prepared from spontaneous adenocarcinomas and passaged into 20 recipients. The primary tumour transplant was removed when ~1 cm³, the spleens of these animals being used at the times after surgery as shown. In all cases cells were lysed with Tris-buffered ammonium chloride and the lymphocyte pool was washed and resuspended in αF₁₀. Various numbers of lymphocytes were then added to 10⁵ ³H-proline-labelled embryo fibroblast target cells.
‡ Arithmetic mean (± s.e.) of cytotoxicity at 24 h to ³H-proline-labelled embryo fibroblasts, using a 50:1 effector:target ratio. The data are arbitrarily normalized to 10% specific lysis of Cef₁₄ by lymphocytes from an 18-day pregnant animal. The data are averaged over independent experiments (all performed with the same batch of frozen target cells), the standard errors thus representing the variation between experiments.

gens are expressed at unique times during embryonic growth, and that subsets of cytotoxic cells develop with reactivity to these antigens. In order to examine in greater detail such specific reactivity to unique embryonic antigens, spleen cells were harvested from animals at different times during pregnancy or tumour growth and triplicate cultures of 5×10⁶ cells were challenged in tissue culture for 5 days with embryonic cells of different ages or semi-allogeneic (C3H×C57BL/6)F₁ spleen cells. Each antigen was presented in a form bound to macrophage monolayers (see Materials and Methods). Following this the spleen cells from individual groups were pooled and harvested, washed in αF₁₀, and tested at various effector:target ratios for their cytotoxicity to ³H-proline-labelled fibroblasts (prepared from embryos of different ages) or to ⁵¹Cr-labelled EL₄ tumour cells. Results of a typical experiment (1 of 4) are shown in Fig. 1.

There are several features of interest in this figure. The data in the 3rd column indicate that all spleen cells responded qualitatively and quantitatively to the same degree to an allogeneic stimulus (as assayed by EL₄ tumour targets). However, for all lymphocytes putatively immunized to embryonic antigens, a source of stimulator/target cells existed which revealed a quantitatively greater response from these lymphocytes than from normal cells. The degree of significance to be attached to the differences documented in this and subsequent Figures is readily appreciated, given that within each experiment the data points have an s.e. ≤8% of the arithmetic mean. Furthermore, the data display once more the specificity suggested in Table I. Thus only cells from late-pregnant animals (primiparous 14/18) and not from virgin or early-pregnant mice were readily stimulated to show cytotoxicity with C₁₈ cells (from 18 day foetuses) (× ... ×). Conversely, cells from early-pregnant mice (primiparous₁₉) were optimally stimulated by C₁₀, though, in contrast to the findings after stimulation with C₁₈ (above), primiparous₁₈ cells were also stimulated to cytotoxicity by C₁₀. One interpretation of these data is that subsets of cytotoxic cells develop with preferential cytotoxicity to antigens which are acquired (and lost) at
unique times in embryo development. Thus primiparous10 lymphocytes react like virgin cells to antigens on C18 (or Cef18, fibroblasts from 18-day foetuses) while primiparous14 lymphocytes contain “memory” cells for C10, C14 and C18.

No less interesting were the data seen with cells from tumour-bearing or tumour-excised animals (last 4 rows of Fig. 1). It was clear that the cytotoxic response from tumour-excised mice was greater than from tumour-bearer. Moreover, cells from the individual tumour groups show signs of the same specificity phenomenon as cells from animals at different times after pregnancy. Thus adeno1 cells were optimally stimulated by C10 and now showed significantly ($P<0.05$) greater cytotoxicity to Cef10 than Cef14. That this is not simply caused by a quantitative difference in expression of one antigen between C10 (Cef10) and C14 (Cef14) cells is indicated by the reverse situation in which adeno2 cells were stimulated significantly more by C14 than by C10, even though almost equivalent cytotoxicity was seen on Cef10 with such stimulated cells.

One interpretation of the data is that there is a difference in antigen expression of at least 2 antigens, one preferentially expressed on C10 (Cef10) and one on C14 (Cef14) each of which can induce subsets of cytotoxic cells preferentially reactive with the different antigens.

**Inhibition of cytotoxicity induced in anti-embryo-immunized cells in culture by target cells bearing the appropriate embryo antigens**

The cross-reactive cytotoxicity generated in the *in vitro*-stimulated cultures above (*e.g.*, in which C14 (●) stimulated cells were often highly cytotoxic to Cef10 targets) might be explained by the presence of cross-reactive antigens on the stimulator/target cells and/or cross-reactive populations of cytotoxic cells. We have investigated this problem by performing “cold competition” experiments in which lymphocytes were sensitized *in vitro* with C10, C14 or C18 and then

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**Fig. 1.**—24 h cytotoxicity of spleen cells from various sources to fibroblasts from embryos of different ages after stimulation of the spleen cells in culture with cells from different age embryo cells. Rows (a) to (h) represent the source of spleen cells. Adeno1 and adeno2 represent spleen cells from mice bearing different adenocarcinomas, and adeno3 resected 10 days, adeno3 resected 20 days, the cells recovered from such mice 20 days after surgical removal of the tumour. All points represent arithmetic means of 3 readings at the effector: target ratio shown. 10^3 $^{35}$H-proline labelled targets and a 24 h test assay were used in all cases except for cytotoxicity to EL4 tumour target cells, which was measured in a 4 h $^{35}$Cr-release assay using 5 x 10^4 $^{35}$Cr-labelled target cells. Data in the columns shown represent the cytotoxicity to the different targets after stimulation with 10 (●), 14 (●) or 18 (●) day embryo cells respectively. The broken line in the third column shows the cytotoxicity to EL4 tumour targets after stimulation with (C3H × C57BL/6)F1 spleen cells (in this the effector: target ratios shown are 2:1, 10:1 and 20:1).
assayed on $^3$H-proline-labelled Cef$_{14}$ in the presence of excess cold C$_{10}$, C$_{14}$ or C$_{18}$. In a “criss-cross” specificity test, cells from the 14-day pregnant animal were also assayed against $^3$H-proline labelled Cef$_{10}$ targets. The data for one experiment (of 3) are shown in Fig. 2. It should be noted that as control experiments we have tested on several occasions the inhibition of cytotoxicity to Cef with unrelated “cold” EL$_4$ tumour target cells, and at no time has any significant perturbation of cytotoxicity been seen. Irrespective of the source of stimulator cells or responder cells, cytotoxicity to Cef$_{14}$ in all cases was optimally inhibited by C$_{14}$ cells (Panels a to d). This was despite the quantitative differences in cytotoxicity seen when different lymphocyte sources were used as responder cells (Panels a to d) or when the source of stimulator cells was varied (rows, Fig. 2). These quantitative differences were similar to those in Fig. 1. When cytotoxicity was measured using Cef$_{10}$ as target, however, the optimum inhibition was seen with C$_{10}$, again irrespective of the source of stimulator cells (Panel e). Taken together, these data argue strongly that the cells causing cytotoxicity represent unique subsets of cells responding to different antigens, which were expressed in different patterns on the various stimulator/target cells used. Thus the cells of primiparous$_{14}$ (P$_{14}$) cytotoxic to Cef$_{14}$ after stimulation by C$_{10}$ (Panel b) were not the same cells assayed when reactivity to Cef$_{10}$ was studied (Panel e). Thus the cross-reactivity seen in Fig. 1 was most likely due to the variable ability of a given stimulator or target to induce or be “killed” by different subsets of cytotoxic cells.

Comparison of the data obtained with adeno$_3$ and adeno$_5$ with the data in the upper two rows (with virgin cells and primiparous$_{14}$ cells respectively) of Fig. 2 and those of Fig. 1 suggest that adeno$_3$ cells were preferentially reactive with an early-appearing embryonic antigen and adeno$_5$ with a later appearing embryo antigen. Cold competition experiments of the type above with cells from tumour-bearing animals gave similar results (unpublished data) supporting the concept of individual subsets of cytotoxic cells.

**Evidence for increased reactivity to embryonic antigens after stimulation with tumour cells**

In an auxiliary test of the similarity of the findings in tumour-related and pregnant mice, we have examined the specificity of cytotoxicity to fibroblast targets of lymphocytes from tumour-resected animals re-stimulated in culture with either

![Fig. 2](image-url)
tumour cells (adeno8 and adeno12, see Fig. 3) or embryonic cells respectively. The data for this experiment are shown in Fig. 3. Similar data have been obtained with other tumours.

Comparison of these data with those of Figs. 1 and 2 led to several interesting conclusions. The quantitative optimum cytotoxicity seen with adeno8 tumour-resected spleen cells was directed against Cef14, and was seen after stimulation with C14 (●) and adeno8 (□) cells. Highly significant reactivity with Cef18 (or Cef10) was only seen after stimulation with autologous tumour cells or late or early embryo cells. These data suggest that adeno8 lymphocytes were preferentially responsive to late-appearing embryonic antigens. By contrast, adeno12 cells were optimally stimulated with C10 (○) or adeno12 (△) cells, gave their optimum reactivity with Cef10, and were poorly reactive with Cef18, even after stimulation with C18. Such data were best explained in terms of a preferential response of adeno12 cells to early-appearing embryonic antigens.

Sedimentation analysis of precursors of cytotoxic cells at different times after exposure to embryonic antigens

A number of workers in recent years have investigated the homologous secondary response in mixed leucocyte cultures in cells challenged previously in vitro or in vivo with histoincompatible cells (Häyry and Anderson, 1975; Hollander, Ginsburg and Feldman, 1974; MacDonald, Cerottini and Brunner, 1974). The data collected from such studies have led to the concept of alternate cycles of blastogenesis (and development of cytotoxic potential) followed by quiescence (and a return to inactive small cells). We have investigated this phenomenon in cell populations which are presumably responding to embryonic antigens. Before re-stimulation of the cells in culture the responding spleen lymphocytes were fractionated by Ig sedimentation to investigate the size of the precursors of cytotoxic cells.

Spleen cells were pooled from a minimum of 2 mice, and 2×10^8 cells were sedimented at 4°C for 4 h in a BSA gradient ranging from 0.3% to 2.0% in PBS. After this time fractions corresponding to cells with differences in sedimentation velocity of 1.0 m/h were collected and the viable cells/fraction counted. No reproducible significant difference in nucleated-cell sedimentation profile in the various groups was noted. The cells were washed, resuspended in αF10 and the primiparous or tumour-related cells were stimulated in culture for 5 days with C14 or adeno17 cells respectively. The spleen cells from the various groups were then harvested, resuspended in αF10 to volumes equivalent to their recovery in the original fractiona-
It is quite apparent from the data of this Figure that changes in the physical size of the precursors of cytotoxic cells for anti-embryo responses did indeed occur, in a manner analogous to anti-allograft responses. The precursor cells in virgin animals have been found to reside in a pool of slow-sedimenting small lymphocytes (2–4.5 m/h—unpublished data). In contrast to this, the precursor cells in pregnant mice (re-stimulated with C14) or tumour-bearing mice (re-stimulated with autologous tumour cells) sedimented with peak velocity 7–10 mm/h (Panel a, Fig. 4). Immediately after birth or tumour resection, there was evidence for 2 populations of precursor cells (Panel b) while at much later times the size of the precursor cells resembled that in virgin animals. As yet, no formal proof is available that the small cell (in virgins) gives rise to the memory large cell (tumour-bearing or P14) which later reverts to a small cell after antigen removal. Nevertheless, the ability to inhibit the development of cytotoxicity in re-stimulated cultures by treatment before culture with anti-Brθ* (Gorczynski, in preparation) and the similarity of much of these data with earlier studies on allo-antigen-primed cells (Häyry and Anderson, 1975) suggest they are indicative of the same phenomenon.

**Evidence for specific inhibition of anti-embryo (not anti-allograft) responses in mice at early times during pregnancy/parturition and tumour growth**

The data above show that the secondary response of tumour-resected lymphocytes to embryo-associated antigens was increased dramatically relative to the response of tumour-bearing cells, and also that the precursors of the cytotoxic cells changed size after tumour removal. The lack of continuity in this size change of precursor cells (see the biphasic distribution of Panel b, Fig. 4) suggested that the enhanced response of tumour-resected cells may in part be due to loss of an in-
hibitory population. Accordingly, we have investigated the ability of cells with sedimentation velocity 4.5–7.0 mm/h (Panel b—Fig. 4) to inhibit anti-embryo or anti-allograft cytotoxic responses.

Spleen cells were pooled from a minimum of 3 animals per group at various times after pregnancy induction or tumour growth, and 2.0×10^8 of each of the pools was sedimented for 3 h at 4°C. From each source the population of cells sedimenting with velocity 4.5–7.0 mm/h was collected, and varying numbers of these cells were mixed with 5×10^6 spleen lymphocytes from a pool of 4 animals at 25–35 days post-partum. The cell mixtures were then sensitized for 5 days in culture with C14 embryonic cells or with (C3H×C57BL/6J) F1 spleen cells. All culture groups were set up in triplicate. After 5 days, the cells within a given group were pooled, and assayed at a constant ratio with 10^3 3H-proline-labelled Cef14 or with 5×10^4 51Cr-labelled tumour target cells (EL4). The ratio used was that representing an effector:target ratio of 80:1 (vs Cef14) or 20:1 (vs EL4) in the control group receiving only the primiparous cells. The data for one experiment of 2 of this type are shown in Table II.

These data support the conclusions drawn from Fig. 4. The sedimented cell population of all spleen cell sources used gave negligible inhibition of the allo- genetic cytotoxic response with 2×10^5 cells per 5×10^6 responder cells, and caused a mean inhibition of some 30% of the control response with 10×10^5 cells—all spleen-cell sources gave about the same inhibition. In contrast, even 2×10^5 cells derived from the spleens of pregnant or tumour-bearing animals, or of animals soon after removal of the source of embryo antigen, caused highly significant (>50%) inhibition of the anti-embryo response (see also Gorczynski, 1976d) but again the maximum inhibition (now 80% to 90%) was seen with cells from pregnant or tumour-bearing animals. At longer times after birth or tumour excision (30 days) this particular sedimented spleen-cell pop-
ulation was no more able to inhibit an anti-embryo response than a similar population prepared from virgin female cells.

DISCUSSION

In earlier studies (Gorcynski, 1976a, d) we reported that virgin mouse lymphocytes spontaneously developed a cytotoxic response in vitro to embryo-associated antigens, that male cells apparently responded in a different fashion from female cells, and that female non-T cells could suppress the development of an anti-embryo response. Given the evidence for some sex-associated differences in the natural frequency of occurrence of a variety of tumours in humans (Silberberg and Grant, 1970), it was thus tempting to speculate that an autoreactive (anti-embryo) response in animals might indeed modulate tumour growth for a tumour expressing embryonic antigens, and that such an effect would be most marked in animals pre-exposed to the embryonic antigen. A necessary corollary of such a hypothesis however is that animals which have been exposed to embryonic antigens (by pregnancy or growth of a tumour expressing embryo-associated antigen) should show an anamnestic response to such antigens different from normal (virgin) cells, perhaps analogous to that recently described by Wekerle (1977) for testicular autoantigen.

In gathering the data described in this report we have attempted to throw light on these questions. In essence it was found (Fig. 1) that, at different times after initiation of pregnancy or induction of tumour growth, differential susceptibility to re-stimulation with cells from embryos of different ages is seen. Lymphocytes from early-pregnant animals responded preferentially to early-embryo cells and vice versa. Tumour-bearer or tumour-resected lymphocytes could also be resolved, in terms of their pattern of reactivity to cells from different-aged embryos (Fig. 1–3) into populations akin to those in early-/late-pregnant animals. Cold competition experiments (Fig. 2) suggested that the most plausible explanation of the data was that cytotoxicity was due to individual clones of cytotoxic cells, each with preferential reactivity to target cells bearing age-related embryonic determinants. Different tumours might then preferentially induce clones of cytotoxic cells which were specific for the embryonic antigen carried by that particular tumour. The data of Fig. 3 show an additional corollary of this hypothesis, in that the pattern of reactivity of cells from tumour-resected animals was analogous to that seen with early-/late-pregnant animals when the cells were re-stimulated with autologous tumour cells or cells from different-aged embryos.

In addition to this evidence for fine specificity discrimination in cells from animals previously exposed to embryonic antigen, evidence was obtained (Fig. 4) that the precursor cells for cytotoxic cells underwent size transitions after embryonic antigen challenge/removal in a manner reminiscent of that seen with lymphocytes from animals primed with cells differing at the MHC (see MacDonald et al., 1974; Häyry and Anderson, 1975; Hollander et al., 1974). Furthermore, both tumour bearer/pregnant animals and animals early after removal of in vivo antigen challenge were found to contain a population of cells with peak sedimentation velocity (4.5–7.0 mm/h) which could preferentially inhibit a response to embryo-associated determinants (relative to the response to allo-antigen determinants)—see Table II. This population was already demonstrable, though to a lesser degree, in virgin females, and may be the same as that previously reported in such animals, and characterized as a non-T-cell population (Gorcynski, 1976d). The relationship of the cytotoxic cell precursors and inhibitory cells in virgin and embryo-antigen-exposed animals, and the effects such cells produce upon adoptive transfer to a tumour-bearing host, remain the topics of future investigation.
The author thanks Ms S. MacRae and Ms F. Sochaiky for their excellent assistance, and Dr Guy Laroye (Department of Pathology, Princess Margaret Hospital, Toronto) for pathological reports on the tumour specimens used. This work has been supported by the Canadian Medical Research Council (Grant MA-5440) and by the National Cancer Institute of Canada.

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