ANTLEUKAEMIA ACTIVITY AS A BYSTANDER EFFECT OF
GRAFT-VERSUS-HOST REACTIONS

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Summary.—The production of graft-versus-host (GVH) reactions in (PVGc × Wis
tar) F1 hybrids by the transfer of PVGc spleen cells resulted in significant resistance of
these recipients to a subsequent challenge with the PVGc leukaemia. Protection
was markedly dependent on dose and timing of allogeneic cell transfer and was
abrogated by irradiation of the cells prior to transfer. GVH activity was shown to
be a prerequisite for induction of the protective effect but was equally effective
when produced by the transfer of Wistar spleen cells in place of PVGc cells.

These points, plus the fact that in vitro investigations of possible immune mechan-
isms failed to demonstrate cytotoxic immunity in treated rats, suggested a non-
specific “bystander” effect as the mechanism of protection. The implications of
such a mechanism are discussed.

The demonstrations of increased immune responsiveness following allogeneic cell interactions (Hamilton, 1973; Katz, 1972) raised the question whether GVH reactions could have a similar effect on immune responses against tu-
mours. Some evidence for this comes from several reports. Medzihradsky
(1966) showed that the induction of a GVH reaction in (Lewis × AVN) F1 rats
given the Walker tumour resulted in prolonged survival times and a decrease
in the number of lethal takes of the tumour. Similar results were obtained
with a methylicholanghrene-induced tumour when a GVH reaction was induced
simultaneously with tumour inoculation (Medzihradsky, Konikova and Novotna,
1973).

In guinea-pigs the transfer of allogeneic lymphoid cells has been shown
to produce resistance to a subsequent inoculum of leukaemia cells. This resis-
tance was manifested by striking pro-
longation of survival times and in some animals which survived indefinitely, re-
sistance to a second challenge with

leukaemia was demonstrable. The effect
was apparently dependent on a GVH
reaction but attempts to determine the
mechanisms involved were unsuccessful
(Ellman et al., 1972; Katz et al., 1972).

In many of the studies involving anti-
tumour effects produced by the transfer of allogeneic cells, it has been difficult
to divorce any direct cytotoxic effects
the allogeneic cells may have on the
tumour from the effects of interaction
of allogeneic cells with those of the
host. We have therefore chosen a GVH
model in which the donor and recipient
are histocompatible with the tumour and
where the effects observed can presumably be attributed solely to the effects of the
allogeneic interaction of lymphoid cells.

We report here our results showing that interaction of lymphoid cells in a GVH
reaction in these circumstances can result in marked inhibition of growth of a highly
malignant leukaemia in rats. We have
further characterized the nature of this
anti-tumour activity and present the
results of initial studies designed to
reveal the mechanism of this effect.

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MATERIALS AND METHODS

Animals.—8–10 week old rats of the inbred PVGc strain were from the MRC laboratories, Carshalton, Surrey, U.K. Wistar strains and the F1, hybrid of this combination with the PVGc were from the University of Sydney animal station.

Tumour.—The leukaemia used in these studies was the PVGc lymphoma kindly supplied by Dr Bruce Roser of the Department of Pathology, University of Sydney. The tumour has a marked leukaemic phase concurrent with lymph node involvement. A highly virulent form of this tumour, obtained by repeated passage in syngeneic animals, was used in these studies. In this form only minimal involvement of lymph nodes is seen and the disease is predominantly that of a rapidly fatal leukaemia. An i.p. dose of 10⁴ leukaemic cells was used in all experiments and produced leukaemia within about 14 days, and death no longer than 10 days after this. No evidence for resistance on the part of susceptible rats (PVGc and PVGc hybrids) has yet been demonstrated (Hersey, 1973a; 1973b).

Tumour cell suspensions were obtained from the peritoneal cavity of previously i.p. infected animals by lavage through a wide-bore perforated needle, with Eagle’s minimal essential medium (MEM) (Microbiological Assoc., Inc., U.S.A., Cat. No. 12-92) containing Penicillin (100 units/ml) and Streptomycin (100 μg/ml).

To determine the day of onset of leukaemia, sequential blood counts of inoculated animals were made on a model D Coulter Counter (Coulter Electronics Ltd., Dunstable, Beds., England). Rats reaching a count higher than 30 × 10⁶/ml of whole blood on two successive days were classed as leukaemic, since all such rats showed progressive increases in blood count from this point on.

Neonatal induction of tolerance.—PVGc rats less than 24 h old were rendered neonatally tolerant of (PVGc × Wistar) F1 by the injection of 8 × 10⁷ hybrid bone marrow cells via the intracardiac route, as described by Grazer (1958).

Tolerance was assessed by a popliteal lymph node weight assay, described by Ford, Burr & Simonsen (1970) for the in vivo detection of GVH reactivity. In this assay 5 × 10⁶ lymphoid cells were injected into the footpads of F1 hybrid rats and 7 days after this, the popliteal lymph nodes were excised and weighed. Lymphoid cells from successfully tolerized PVGc rats produced no increase in node weight while normal PVGc cells produced a 4- to 5-fold increase in weight.

In vitro ⁵¹Cr release assays

⁵¹Cr labelling of target cells.—Approximately 3 × 10⁶ tumour cells in 1 ml of MEM supplemented with 10% Foetal Bovine Serum (FBS) (Australian Laboratory Services, Batch 28) were incubated with 100 μCi of Na₂ ⁵¹CrO₄ (Radiochemical Centre, Amersham, Bucks., U.K.) for 2 h at 37°C. The cells were washed twice in 25 ml of MEM and resuspended in MEM + 10% FBS buffered with 20 mM of Heps.

Antibody-dependent (AD) cell-mediated cytotoxicity.—Assay of rat sera for this activity was carried out as previously described (Hersey, 1973b) using human effector cells obtained by Hypaque/Ficoll separation of peripheral blood. 6 × 10⁶ effector cells in 0·2 ml were used in each assay and 2 × 10⁴ target cells were added in 0·2 ml. Test sera from rats were added in serial concentrations in duplicate in a volume of 0·05 ml. The total volume of 0·45 ml was incubated in capped round-bottomed tubes for 6 h at 37°C. After this the cells were sedimented by centrifugation and 0·2 ml of each supernatant was transferred to adjacent tubes for counting.

Direct cell-mediated cytotoxicity.—Spleen cells were assayed for direct cytotoxicity activity after depletion of macrophages by glass absorption (Hersey, 1973a). Absorbed spleen cells at concentrations of 3 × 10⁸, 1 × 10⁸ and 3 × 10⁷ in 0·5 ml were added to target cells (2 × 10⁴ in 0·5 ml). Incubation was for 6 h at 37°C in capped flat-bottomed tubes. After incubation the cells were centrifuged and 0·5 ml of supernatant transferred to adjacent tubes for counting. All counting was done on a Wallac Gamma Sample Counter.

Percent ⁵¹Cr release was calculated as follows:

\[ \% \text{ } ⁵¹\text{Cr release} = \frac{a \times 2.25}{a + b} \times 100 \]

where \( a \) = activity in supernatant tube and \( b \) = activity in tube with cell pellet and remaining supernatant. For the direct cytoto-
toxic assay, the factor was 2 in place of 2.25.

In vitro assay for cytostatic serum factors.—Spontaneous \( ^{3}H \)-thymidine uptake by leukaemic cells was assessed by short-term culture of \( 3 \times 10^{6} \) cells in a volume of 1 ml of Dulbecco's modified Eagle's medium (DEM), supplemented with 10% FBS, penicillin and streptomycin. Cultures were pulsed for 4 h with 2 \( \mu \)Ci of isotope at specific activity of 5 Ci/mM (Radiochemical Centre, Amersham) 2 h after the cultures were set up. Test and control serum was added in varying dilutions to these tubes at the time of dispensing to determine whether such sera could produce a decrease in isotope uptake. Harvesting and counting were carried out by standard trichloroacetic acid extraction and \( \beta \) scintillation counting procedures.

Experimental protocol.—Spleen cells were taken from parental PVG\(_{e}\) rats (except where otherwise indicated) approximately 2 months old, and injected slowly i.v. into \( F_{1} \) rats 6–8 weeks of age at the times indicated in the text, in relation to the tumour inoculum of \( 10^{4} \) cells given i.p. on Day 0. Controls in each experiment were rats given tumour only or in some experiments rats given tumour and \( F_{1} \) spleen cells at the same doses as the PVG\(_{e}\) spleen cells. Leukaemia onset was determined by sequential count of blood from the tail veins.

Statistical analysis.—Wilcoxon's two sample rank test was employed to assess the significance of variations in the day of leukaemia onset between groups of 5–6 rats. Probability levels of 5% were chosen as significant.

Irradiation.—Spleen cells were irradiated by a \( ^{60}Co \) source at a distance of 12 inches in a plane vertical to the beam axis on a rotating platform. Dose rate was 50 rad/min.

RESULTS

The protective effect of PVG\(_{e}\) spleen cells.—(Figure 1.) To determine whether PVG\(_{e}\) spleen cells could produce protection from leukaemia in (PVG\(_{e}\) × Wistar) \( F_{1} \) rats, spleen cells in varying numbers were transferred i.v. to \( F_{1} \) rats 6 days before these rats received an i.p. inoculation of \( 10^{4} \) tumour cells. The time of onset of leukaemia in these rats was determined and compared with that of a group which had received \( 10^{8} \) syngeneic (\( F_{1} \)) cells on Day —6.

Rats pretreated with \( 3 \times 10^{7} \) or \( 10^{8} \) PVG\(_{e}\) spleen cells showed significant delays in leukaemia onset when compared with the control group \( (P < 0.01) \). Recipients of \( 3 \times 10^{8} \) PVG\(_{e}\) spleen cells showed no delay in onset.

![Figure 1](image-url)

**Figure 1.**—Results show % of \( F_{1} \) rats in each group with leukaemia on sequential days after leukaemia inoculation. Group 1 rats received \( 10^{8} \) \( F_{1} \) spleen cells on Day —6; groups 2, 3 and 4 received \( 3 \times 10^{7}, 3 \times 10^{8} \) and \( 10^{8} \) PVG\(_{e}\) spleen cells respectively, on Day —6. All rats were inoculated with \( 10^{4} \) leukaemic cells on Day 0.
The effect of irradiation (1000 r) of 
PVGc spleen cells before transfer.—(Table I.) Using the optimal conditions for 
protection, a transfer of 10⁸ PVGc spleen 
into F₁ hybrids produced a significant 
delay in leukaemia onset. When, how-
ever, such cells were pretreated with 
1000 r, no protective effect was observed. 
Since it was felt that irradiated cells 
might be more rapidly cleared from the 
circulation and hence be prejudiced in 
their protective potential, a group of 
rats received 3 doses of 10⁸ irradiated 
cells on Days —6, 0 and +6 (with leuka-
emia challenge on Day 0). This pro-
duced a significant delay in onset com-
pared with recipients of leukaemia cells 
only (P < 0.05). However, the degree 
of protection achieved with 3 doses of 
irradiated cells was significantly less than 
that produced by either 1 or 3 doses of 
unirradiated cells (P < 0.01).

The effect of Wistar spleen cells on 
leukaemia onset.—(Table II.) To test 
whether protection was due to immuniza-
tion of F₁ animals against antigens 
cross-reacting between PVGc spleen cells 
and leukaemia cells (i.e. strain specific), 
Wistar spleen cells were used instead 
of PVGc spleen cells. As shown in 
Table II, when 10⁸ Wistar spleen cells 
were given on Day —6 in relation to 
the tumour inoculum, a significant delay 
in onset of leukaemia was observed 
(P < 0.01) suggesting that allogeneic in-

\[ \text{Table I. The Effect of Irradiation of Transfused Syngeneic Donor Cells on their Anti-leukaemic Effect} \]

| Group | Day of leukaemia onset | P value vs. Gp. 1 |
|-------|------------------------|------------------|
| 1. Turnour only | 13, 13, 14, 14, 14 | -- |
| 2. 10⁸ cells | 16, 16, 16, 17, 17, 18 | <0.01 |
| 3. 10⁸ cells x 3* | 18, 19, 19, 20, 20 | <0.01 |
| 4. 10⁸ irr. cells | 14, 14, 15, 15, 15 | NS |
| 5. 10⁸ irr. cells x 3* | 14, 14, 15, 15, 16 | <0.05 |

* On Days —6, 0, and +6 in relation to inoculum of 10⁴ tumour cells on Day 0.
† Greater than Group 5 (P < 0.02).
interaction of the cells was responsible for the effect.

The effect of F1 spleen cells on leukaemia onset in PVGc rats.—Since protection was apparently dependent on an allogeneic cell interaction it was necessary to assess whether protection could be provided by the interaction of Parent and F1 cells in a HVG situation. When 10^8 F1 hybrid spleen cells were transferred to PVGc rats 6 days before leukaemic inoculation there was no significant difference between the time of leukaemia onset in these rats and in rats which received tumour cells only.

The effect of tolerant PVGc spleen cells.—(Figure 3.) The dependence of protection upon a GVH reaction was further investigated by assessing the effect of spleen cells from PVGc rats which had been rendered neonatally tolerant of (PVGc x Wistar) F1 and shown to be incapable of inducing a GVH response in vivo.

Rats given 10^8 tolerant spleen cells on Day -6 and 10^4 leukaemia cells on Day 0, failed to demonstrate a delay in onset of leukaemia, although 10^8 normal cells produced significant delay (P < 0-01). As a further control in this experiment a group of rats received 10^8 "non-tolerant" cells from PVGc rats in which tolerance induction had failed and which were therefore able to produce GVH reactions in the in vivo GVH assay. As shown in Fig. 3 recipients of such cells showed a significant degree of delay, similar to that produced by normal PVGc cells.

**AD cell-mediated cytotoxicity.**—(Figure 4.) Groups of F1 rats were given 10^8 PVGc spleen cells on Day -6 and serum samples taken on Days 0, +6 and +12 in relation to the tumour inoculum. Samples from 3 individual rats were assayed at these time periods for evidence of AD cell-mediated cytotoxic activity and as shown in Fig. 4 no evidence of this

### Table II.—Effect of Transfer of Allogeneic Donor Cells on Leukaemia Onset

| Group | Day of leukaemia onset | P value 1 vs. 2 |
|-------|------------------------|-----------------|
| 1. Tumour only | 15, 15, 15, 16, 16 | — |
| 2. 10^8 Wistar spleen cells Day -6 | 17, 18, 18, 19, 24, 25 | <0.01 |

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**Fig. 3.**—All groups of F1 rats received 10^4 PVGc leukaemia cells on Day 0. In addition, on Day -6, group 1 rats received 10^8 "tolerant" PVGc spleen cells; group 3, 10^8 "unsuccessfully tolerated" PVGc spleen cells (see text); group 4, 10^8 normal PVGc spleen cells. Group 2 received no spleen cells.
activity was found. Sera from Wistar rats immunized with $10^8$ PVG<sub>e</sub> lymphoma cells were included in the assay as a positive control and indicate the sensitivity of the assay.

**Direct cell-mediated cytotoxicity.**—(Fig. 5.) Direct cell killing was assessed in macrophage-depleted spleen populations from F<sub>1</sub> rats treated with $10^8$ PVG<sub>e</sub> spleen cells on Day -6. Rats were sacrificed at Days 0, +6 and +14 and their spleens assayed for the presence of cytotoxic activity against the leukaemia cells. No significant levels of cytotoxicity were demonstrated at these times. Graph (d) represents the level of cytotoxicity developed in Wistar rats inoculated with PVG<sub>e</sub> lymphoma 5 days before assay.

**Cytostatic serum factors.**—Leukaemic cells were found to incorporate spontaneously a reproducible level of $^3$H-thymidine under short-term culture conditions. Sera from experimental rats were added in varying dilutions to see whether they could exert an inhibitory effect on isotope uptake. When the effect of such sera was compared with the effect of sera from normal rats, no significant depression of isotope uptake could be demonstrated at any time during the GVH protocol. It was, however, found that all sera from normal or test rats had a markedly inhibitory effect on isotope uptake by the leukaemic cells.

**DISCUSSION**

The use of allogeneic lymphoid cells in immunotherapy of leukaemia has been reported by several workers (Woodruff and Nolan, 1963; Mathé et al., 1967; Boranić, 1968; Britton, 1972; Bortin, 1974). The anti-leukaemia effect in these studies has generally been regarded as an example of adoptive immunotherapy, resulting from histocompatibility differ-
Fig. 5.—Lymphocyte cytotoxicity assay of splenic cells from F₁ rats undergoing GVH protocol and tumour inoculation. Spleens taken at (a) Day 0; (b) Day +6; (c) Day +14; (d) cytotoxic control (see text).

ence between donor lymphoid cells and the tumour of the recipient. In contradiction, the data presented here demonstrate an anti-leukaemic effect in a GVH reaction in which both donor and recipient are syngeneic with the leukaemia. The anti-leukaemic effect, in terms of delay in the time of leukaemia onset, was equivalent to a 3–4 log reduction in the size of the initial $10^4$ leukaemia cell inoculum even though both PVGc and the F₁ hybrid are incapable of rejecting inoculations of very small numbers of these tumour cells (Hersey, 1973c).

Over the past few years several immunological effects of allogeneic cell interactions have been described, other than the anti-tumour effects described here. Increased antibody production after primary and secondary antigenic challenge has been described and referred to as the “allogeneic effect” (Katz, 1972; Hamilton, 1973). Abrogation of tolerance has also been described under similar conditions by McCullagh (1970). Similarities of the anti-tumour effect to these later phenomena are apparent. The timing of the GVH reaction in relation to the challenge with tumour cells or antigen has been found critical in all studies and coincides with the time of peak GVH activity. Similarly the dosage of cells given to the recipients has also been shown to be important so that, in our own studies, if the parental cell inoculum was either too large or too small the effect was abrogated. Also, as in our own studies, irradiation of the donor cells rendered them much less effective in producing the described effect suggesting that viable cells capable of multiplication were needed.

In view of the similarities noted with these other described effects of allogeneic cell interactions, the question arises to what extent they can be implicated in the anti-tumour effect described in this model. To answer this question we have carried out in vitro studies to detect either direct cell-mediated or AD cell-mediated immunity. Our failure to find any evidence of cytotoxic immunity by the assays argues against specific immune mechanisms being involved but cannot be regarded as definitely excluding them.
The assays used have been shown by previous studies (Hersey, 1973a, b), and by the controls used in the present study to be sensitive in detection of these mechanisms after allogeneic immunizations but could, nevertheless be ineffective in the semi-syngeneic model used here.

Another possible mechanism of the anti-tumour effect considered in this study was that the F_1 recipients recognized antigens on the PVG<sub>c</sub> spleen cells and were in effect being immunized against similar antigens on the PVG<sub>c</sub> leukaemia. Although this contradicts classical transplantation dogma, the concept of anti-parental responses receives some support from the literature (Cudcowicz and Stimpfling, 1964; Field, Cauchi and Gibbs, 1967; Ramseier and Lindenmann, 1969).

Several of the experiments described in this study argue strongly against this possibility. Firstly it was shown that PVG<sub>c</sub> cells tolerant to the F_1 recipient did not give rise to any anti-tumour effects. As it seems likely that the tolerant cells have the same antigens as non-tolerant cells this argues against the effect being a result of recognition of parental antigens. Similarly the experiments using irradiated spleen cells can be interpreted in the same way. Even when 3 times the dose of irradiated cells was given the anti-leukaemic effect was still much less than that resulting from a single dose of unirradiated spleen cells. Secondly the kinetics of the protection argues against an immunization effect, in that cells given 14 or 21 days before could be expected to provide a similar immunization to that at 6 days. For further evidence against this mechanism the experiment using Wistar spleen cells can be cited. If immunization against PVG<sub>c</sub> spleen cells was involved, then Wistar spleen cells could be expected to be less effective than PVG<sub>c</sub> spleen cells. This was found not to be the case and equal protection was afforded by Wistar spleen cells.

We therefore consider the most likely explanation for the observed anti-tumour effect is that it results from non-specific cytotoxic activity generated during the course of the GVH reaction. Non-specific cytotoxic effects of GVH activity have been suggested by the in vivo studies of Elkins and Guttmann (1968) and Streilein and Billingham (1970). In vivo analyses of such effects by Singh, Saba- dini and Sehan (1971, 1972) showed that non-specific cytotoxic effects were generated 4-8 days after parental cell transfer and were mediated by host cells. More recent demonstrations of similar effects were made by Britton (1974) who found evidence of in vivo cytotoxicity against syngeneic lymphoma cells in mice at the time of peak GVH activity.

If our interpretation is correct it is clear that "non-specific" cytotoxic mechanisms may play an effective role against tumour cells in vivo and may underlie the protective effect seen in several immunotherapy procedures involving transfer of allogeneic cells. If so, the measurement of non-specific cytotoxicity against tumour cells may be of equal importance to that of specific immune mechanisms in the evaluation of host resistance to tumours and in the development of immunotherapy procedures.

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