IMPROVED CHIMAERIC MOUSE MODEL CONFIRMS THAT RESIDENT PERITONEAL MACROPHAGES ARE DERIVED SOLELY FROM BONE MARROW PRECURSORS

A. R. MURCH, MIRANDA D. GROUNDS AND J. M. PAPADIMITRIOU

Department of Pathology, University of Western Australia, Queen Elizabeth II Medical Centre, Nedlands 6009, Western Australia, Australia

SUMMARY

The origin of resident peritoneal macrophages was studied in radiation mouse chimaeras with and without reconstitution of the peritoneum with viable isogeneic peritoneal cells. The selection of host and donor strains were such that the isoenzymes of glucose-6-phosphate isomerase could be used to distinguish host from donor bone marrow derived cells. It was found that the resident peritoneal macrophages were completely replaced by bone marrow donor derived cells within 5–6 weeks. There was little difference between the results from mice which had been additionally reconstituted with peritoneal cells and those which were not.

KEY WORDS—Peritoneal macrophages, radiation mouse chimaeras.

INTRODUCTION

While there is general agreement that exudate peritoneal macrophages are derived directly from circulating monocytes,[1,2] the origin of resident peritoneal macrophages is still in question. Two theories have been proposed: one suggests that resident peritoneal macrophages form a separate, self-replicating population (see Daems[2] for a review), while the other proposes that resident macrophages, like exudate macrophages, originate in the bone marrow, and are continuously replenished from that source (see van Furth[1] for a review).

The use of radiation chimaeras to study the origin of peritoneal macrophages[3–6] has provided strong support for the theory of bone marrow origin. In these experiments the bone marrow of irradiated hosts was replaced with donor marrow from a different strain. In the peritoneum of such chimaeras donor macrophages steadily replaced resident host macrophages over a period of about 6 weeks, until finally only donor macrophages could be identified in the resident population. The weakness of these experiments is that any potential population of dividing host cells in the peritoneum will have been destroyed by irradiation. In addition, cell markers used to distinguish host from donor macrophages in these studies were not entirely satisfactory: Virolainen[5] used the cytogenetic marker, T6, which can only differentiate between dividing cells and thus is selective; Balner,[3] and Goodman[4] distinguished cells from two strains of mice using the cytotoxic activity of specific antisera where nonspecific death of some cells can be a complicating factor, and Haller et al.[6] used differences in genetically determined susceptibility to murine influenza virus.

In the present experiments the criticisms of the radiation chimaera model are overcome by replacing irradiated host peritoneal cells with healthy isogeneic peritoneal cells and a different cell marker is employed. The mouse strains used were each homozygous for one of the two isoenzyme

forms of glucose-6-phosphate isomerase (GPI; E.C. 5.2.1.9) which enables cells of the two strains to be identified by distinct isoenzyme mobilities on starch gel electrophoresis. GPI isoenzymes are effective markers for the nuclei which code for them and have been widely used as cells markers.[7,8] By injecting donor bone marrow cells from animals homozygous for one GPI isoenzyme into host animals homozygous for the other form, it was possible to determine whether resident peritoneal macrophages are an independent self-perpetuating cell population or are replaced by circulating bone marrow derived cells.

METHODS

The mouse strains C3H (homozygous for the electrophoretically fast GPI isoenzyme) and AKR (homozygous for the slow isoenzyme) were maintained as inbred colonies: these strains are compatible (H-2k) at the H-2 locus.[9] All experimental animals were 10–12-week-old males.

Whole body irradiation (800 rads, Siemens deep x-ray; 200 kV; 1 mm Cu half value layer; 0·5 Cu filter; 83·2 rad/min) was given to unanaesthetized mice in a lidded Perspex box. Experimental animals were divided into two groups designated A and B: group A had only bone marrow reconstitution, whereas group B had bone marrow and peritoneal cell reconstitution. AKR bone marrow cells (and in mice of group B also C3H peritoneal cells) were injected within four hours of irradiation. Marrow cells were obtained from mice by syringing thoroughly cleaned femurs with phosphate buffered saline, pH 7·1 (PBS). Cells were washed once with PBS, passed through Miracloth (Calbiochem) to remove clumps, and approximately 5×10^6 cells in 0·2 ml PBS injected into tail veins of irradiated C3H mice.

Peritoneal cells, rich in resident macrophages (typically 60 per cent macrophages and 35 per cent lymphoid cells[10]) were obtained by washing peritoneal cavities of C3H mice with 2 ml PBS. Cells were concentrated by centrifugation, and injected in 0·5 ml PBS into the peritoneal cavity of C3H mice (group B). Cells from two donor mice were used for each host (approximately $2–4 \times 10^6$ cells). Poor survival of many mice beyond 20 days was attributed to mild 'graft versus graft' disease, resulting from C3H lymphocytes in the injected peritoneal washings interfering with reconstitution by AKR marrow cells. To circumvent this problem,

another group of 10 mice (designated B* in Table II) were injected with a purified population of peritoneal macrophages. Peritoneal washings were settled in a Petri dish for 2 h, adherent macrophages washed by pipetting PBS vigorously over the surface of the dish to remove loosely attached lymphocytes, then macrophages scraped off with a rubber policeman, washed and concentrated by centrifugation for injection as above. Dye exclusion tests using trypan blue showed that approximately 40 per cent of the macrophages obtained this way were viable. Each animal received approximately 2×10^5 viable macrophages.

At varying times after irradiation, mice were killed by cervical dislocation. To obtain bone marrow cells, one femur was thoroughly cleaned of adherent tissue, cut longitudinally, and the marrow scraped out directly on to a small piece of Whatman No. 1 filter paper (approximately 1×5 mm). Peritoneal cells were lavaged with 2 ml PBS and in many instances peritoneal cells from paired mice were pooled. Cells were pelleted, resuspended in culture medium consisting of MEM (Flow Laboratories, Sydney) + 10 per cent fetal calf serum (Flow Laboratories, Sydney), and allowed to settle in 35 mm Petri dishes at 37°C for either 4 h or overnight. The supernatant was pipetted vigorously over the surface to remove loosely attached cells, spun, and this cell pellet collected on to a piece of filter paper. Peritoneal macrophages adhering to the Petri dish[11] were rinsed twice with PBS, scraped off with a rubber policeman, spun and the pellet collected on to a piece of filter paper. Samples were stored in sealed containers at −20°C.

Pieces of filter paper were inserted directly into an 11 per cent starch gel, and electrophoretic separation and visualization of GPI isoenzyme bands was performed as described by Grounds et al.[12] The relative proportions of fast and slow isoenzyme bands were visually graded and band size indicated on a scale from (+) to + + + + + ; where no isoenzyme band was detected, this was indicated by −.

A control experiment was set up to determine whether injected macrophages remained in the peritoneum or were rapidly dispersed or destroyed. Three C3H mice were irradiated as in the other experiments, and approximately 2×10^5 settled AKR macrophages were injected into the peritoneum. At 6 days (before the mice began to die of radiation effects) the peritoneal cells were collected, and macrophages separated for electrophoresis as before.

RESULTS

Isoenzyme patterns of mice were examined from 3 to 61 days after irradiation. The results of 37 mice from five experiments are shown in Tables I and II: 24 mice (group A) were reconstituted with AKR bone marrow (Table I), and 13 mice (group B) with AKR bone marrow and C3H peritoneal cells (Table II).

Bone marrow

GPI isoenzyme patterns of irradiated C3H bone marrow reconstituted with AKR cells were similar in all mice (Tables I and II). AKR isoenzyme was not detected at day 3, but was present by day 5 (Fig. 1, A_3). The proportion of AKR isoenzyme increased rapidly and was usually equal to, or greater than that of host C3H isoenzyme by day 7 (Fig. 1, A_5). From day 7 the small amount of host isoenzyme present in bone marrow samples (Fig. 1, B_8) was attributed to persisting host red blood cells

which, unlike white blood cells, are known to survive for several months after irradiation.[13]

Non-adherent peritoneal cells

Non-adherent cells from the peritoneum of mice are largely lymphocytes (85 per cent) with occasional polymorphs, mast cells and degenerate mesothelial cells.[10] In group A, where peritoneal cells were not replaced after irradiation, bone marrow derived AKR isoenzyme was not detected in samples of non-adherent cells examined at 3 or 5 days, and only a trace amount was visible in one of two samples examined at day 7. However, by day 15, AKR isoenzyme predominated. The small proportion of host isoenzyme present in samples after this time was probably due to contaminating red blood cells since these were always seen in peritoneal washings. Host C3H isoenzyme had almost disappeared by day 49.

In group B, where mice were reconstituted with C3H peritoneal cells, isoenzyme patterns similar to

Table I—(Group A) Isoenzyme results of irradiated C3H mice reconstituted with AKR bone marrow

		GPI isoenzyme patterns					
Mouse	Days after irradiation	Bone marrow		Non-adherent peritoneal cells		Peritoneal macrophages	
		C3H	AKR	C3H	AKR	C3H	AKR
*A_1	3	+ + +	−				
A_1	3	+ + +	−	+	−	−	−
*A_2	5	+ +	+				
A_2	5	+ +	+	+ + +	−	+	−
*A_3	5	+ +	+				
A_3	5	+ + +	+	+ + +	−	−	−
A_4	7	+	+ +	+	−	+	(+)
*A_5	7	+ +	+ +				
A_5	7	+ + +	+ + +	+ +	(+)	+ +	−
A_6	9	+	+ +	+ +	+	(+)	−
*A_7	10	+ +	+ +				
A_7	10	+	+ + +	+ +	+	+ +	+
A_8	12	+	+ + +	+	+	+	+
A_9	15	+	+ + +	(+)	+ +	(+)	+
A_{10}	21	+	+ + +	(+)	+	+	+ +
*A_{11}	24	+	+ + +				
A_{11}	24	+	+ + +	+	+ + +	+	+ + +
A_{12}	31	+ +	+ + + + +	−	+	+	+ + +
A_{13}	35	+	+ + + +	+	+ + +	−	+ + +
A_{14}	38	+ +	+ + + + +	−	+	−	+ + +
*A_{15}	49	+ +	+ + +				
A_{15}	49	+	+ +	(+)	+ + +	−	−
*A_{16}	61	+	+ +				
A_{16}	61	−	+ +	−	+ +	−	(+)

*In these samples peritoneal cells from two mice were pooled to increase isoenzyme detection in macrophages and non-adherent cells. Bone marrow samples from the individual mice were run separately.

Table II—(Group B) Isoenzyme results or irradiated C3H mice reconstituted with AKR bone marrow and with C3H peritoneal cells

| Mouse | Days after irradiation | GPI isoenzyme patterns | | | | | |
| | | Bone marrow | | Non-adherent peritoneal cells | | Peritoneal macrophages | |
		C3H	AKR	C3H	AKR	C3H	AKR
B_1	7	+ +	+ + +	+	(+)	+ +	+
B_2	9	+ +	+ + +	+ +	+ +	(+)	−
B_3	12	+ +	+ + + +	+	+	+ + +	+ + +
B_4	15	+	+ + + +	+ +	+ +	+ +	+ +
B_5	20	+ +	+ + + +	+ +	+ +	+	+
B_6*	21	+ +	+ + + +	+	+ +	+	+ +
B_7	34	+	+ + +	+ +	+ +	+ +	+ +
B_8	35	(+)	+ + + + +	+ +	+ + +	+	+ + +
B_9	40	+ +	+ + + +	+ +	+ + +	−	+ + +
B_{10}*	42	+ +	+ + + + +	(+)	+ +	+	+ + +
B_{11}	47	+ +	+ + + + +	+	+ + +	−	+ + +
B_{12}*	49	+	+ + + + +	+	+ +	−	+
B_{13}*	56	+	+ + + + +	(+)	+ + +	−	+ +

* Series B with purified peritoneal macrophages.

group A were found up to day 12. From day 15 the proportion of C3H isoenzyme was higher than in group A (Fig. 1, day 35 cf. B_8 with A_{13}) and this was attributed directly to implanted C3H cells, particularly lymphocytes. The poor survival of mice in this series beyond day 20 was probably also due to these viable C3H lymphocytes resulting in mild 'graft versus graft' reaction. In group B* where mice were reconstituted with purified peritoneal macrophages containing few or no lymphocytes, there were few deaths, and samples of non-adherent cells contained a smaller proportion of C3H isoenzyme (Table II, cf. B_6 with B_5) and were similar to group A (cf. Tables II and I, B_6 and A_{10}).

Peritoneal macrophages

More than 90 per cent of peritoneal cells adhering to a Petri dish are macrophages.[11] Bone marrow derived AKR macrophages were not detected in the peritoneum before day 7 and even at this time were not found in one of three samples. In some early samples of peritoneal macrophages little or no GPI activity was detected, which was the result of excessive lysing of macrophages and consequent loss of enzyme during collection. This was corrected by more gentle cell collection. In group A, the proportion of AKR isoenzyme was greater than C3H isoenzyme from day 15. A small amount of C3H isoenzyme was detected up to day 31, but by day 35 only AKR isoenzyme was present (Fig. 1, A_{13} cf. B_8), indicating that irradiated C3H macrophages do not survive beyond this time.

In group B, where peritoneal macrophages were replenished with viable C3H cells, the proportion of C3H macrophages was high, as expected. In contrast to group A, similar amounts of C3H and AKR isoenzyme were present from days 12 to 34, although, there were only traces of C3H isoenzyme in samples collected after this time. The time of disappearance varied slightly between animals: though not detected in samples taken at day 40, 47, or 49, C3H isoenzyme was present in one sample at day 42 (Table II).

In control C3H mice injected with purified AKR peritoneal macrophages but without AKR marrow reconstitution, peritoneal macrophages collected 6 days after transplantation showed equal proportions of AKR and C3H GPI isoenzyme indicating that donor macrophages remained in the peritoneum for at least this period.

DISCUSSION

The results show that in both groups of mice (A and B) there is steady replacement of C3H peritoneal macrophages by bone marrow derived AKR cells from about day 7 (when marrow transplants begin to take) until day 35 (Group A) or after day 42 (Group B) when only AKR cells were detec-

Bone marrow

−ve

++ +++ (+) C3H

+ +++ ++++ AKR

Origin

Day 5   Day 7   Day 35
A₃   A₅   B₈

Non-adherent peritoneal cells

+ ++

+++ +++

Day 35   Day 35
A₁₃   B₈

Peritoneal macrophages

++ − +

− +++ +++

Day 7   Day 35   Day 35
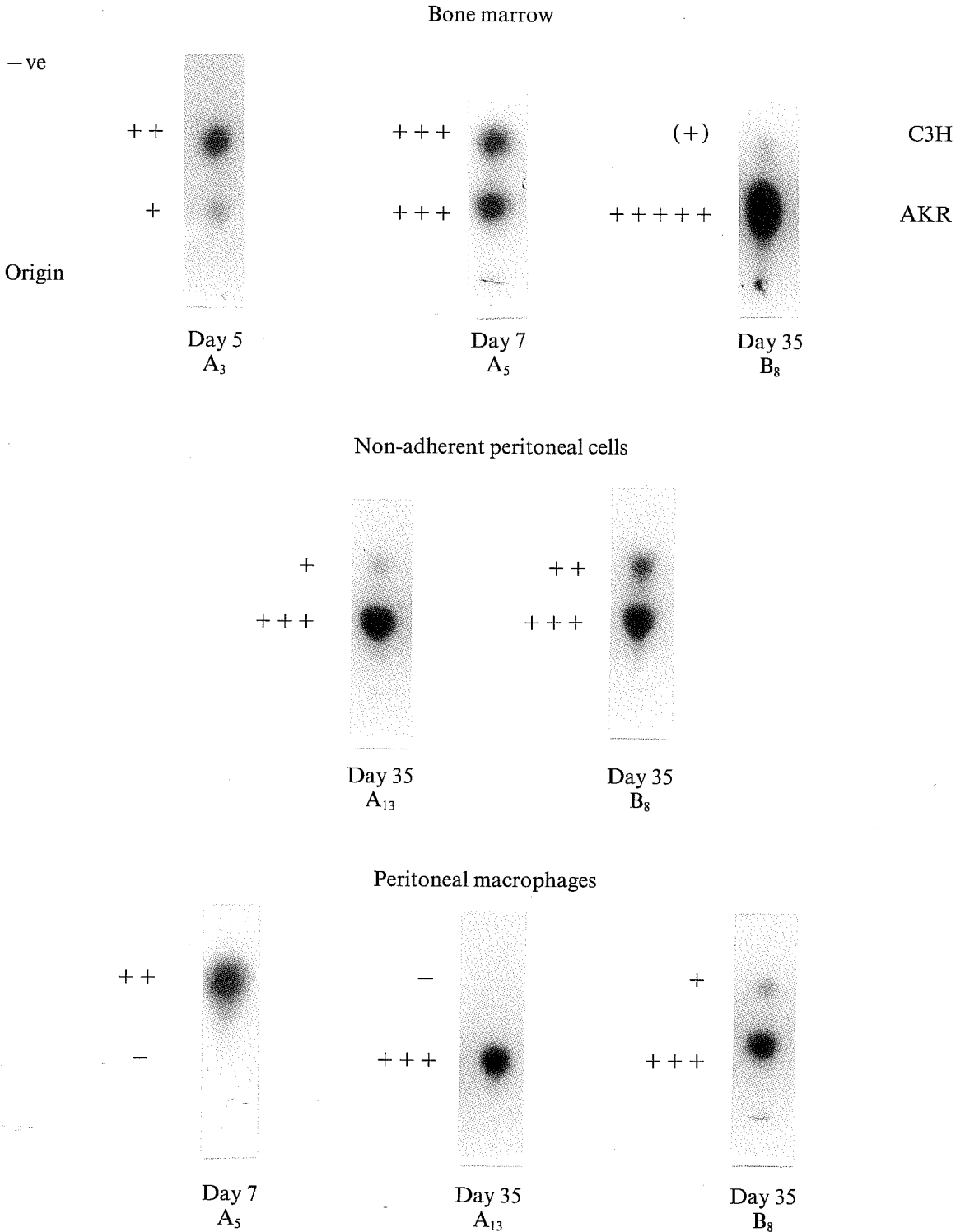A₅   A₁₃   B₈

Fig. 1—Starch gel electrophoresis patterns of GPI isoenzymes. (See Tables I and II and text for details)

ted. The disappearance of C3H peritoneal macrophages in Group B, where healthy peritoneal cells were injected into the peritoneum following irradiation, shows that dividing cells of the peritoneum do not significantly contribute to this population of resident macrophages. This is an important finding since previous work using radiation chimaeras (see Introduction) can be criticized on the basis that the capacity of possible host macrophage precursors in the peritoneum to divide would have been destroyed by irradiation. One possible objection to the present experiments is that an inadequate number of cells may have been used to repopulate the irradiated peritoneum. The total number of cells in the murine peritoneum in the normal steady state is not known, nor is the proportion of progenitor cells (if any), but the $2-4 \times 10^6$ cells injected in the present experiments closely approximates the number of cells which can be obtained by thorough washing of the peritoneum.[2] Even if the number of precursor cells reinjected is significantly lower than the number present in the normal steady state, they would still be expected to contribute to the repopulation of the peritoneum. Moreover, the electrophoretic technique used is sensitive enough to detect even a very small contribution. That the transplanted peritoneal macrophages remain viable in the peritoneum for at least 6 days is demonstrated by the significant amounts of AKR isoenzyme detected in irradiated C3H mice injected with AKR peritoneal macrophages but without AKR bone marrow transplants. The disappearance of C3H peritoneal macrophages by day 35 in group A compared to about day 42 in group B suggests that irradiation shortens the life of resident macrophages.

Our results lend no support to the idea that macrophages resident in the peritoneal cavity are a predominantly self-replenishing population (review, Daems[2]). It should be noted that the use of radiation chimaeras does leave open the possibility that resident macrophages might derive from stem cells lying within tissues of the peritoneum, such as the omentum milky spots: any contribution of such dividing cells would be prevented by irradiation and would not be overcome by reconstitution with healthy peritoneal washings. Available evidence suggests that milky spot cells which give rise to resident peritoneal macrophages, are in turn derived from blood-borne monocytes from the marrow, however, the possibility of a milky spot macrophage stem cell has not been excluded.[14,15]

In conclusion, these results confirm those of Balner[3] and show that in the mouse, resident peritoneal macrophages are of bone marrow origin, and that they turn over completely within a period of 5–6 weeks.

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