THE ROUTE OF IMMUNIZATION IN TRANSPLANTATION IMMUNITY

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IT is commonly believed that all routes of administration of homologous tissue cells are equally effective for the elicitation of transplantation immunity. That this view needs at least some qualification was made clear by Medawar's (1946*a*) finding that suspensions of leucocytes are very much more effective in inducing immunity in the rabbit when injected intradermally rather than intravenously. This peculiarity of the intravenous route in rabbits also extends to suspensions of epidermal cells, for it has been reported by Billingham and Sparrow (1955) that a dosage of epidermal cells sufficient to elicit a powerful immunity if injected intradermally, fails to elicit any detectable immune response when injected intravenously. Indeed, a significant *prolongation* of the survival time of test skin homografts obtained from the cell donor was usually the outcome.

The subject matter of this paper is a comparative study of the immunological consequences of injecting homologous cells by various routes in the three most common laboratory mammals : mice, rabbits and guinea-pigs.

MATERIALS AND METHODS

Principle of the Experiments

A state of transplantation immunity, or heightened resistance to homografts, cannot at present be identified satisfactorily by any *in vitro* test. It must therefore be identified by following the fate of test grafts transplanted to a putatively immune subject from the donor whose cells it had previously received, or from another donor of similar genetic constitution. The most convenient test grafts are furnished by skin, or by portions of a transplantable tumour indigenous to the donor strain.

When a skin homograft is transplanted to an immune host it undergoes a more rapid destruction than when transplanted to a normal one, and it never enters that transient proliferative phase which is characteristic of grafts on untreated animals. A state of immunity may therefore be diagnosed according to the histological appearance of a test skin graft biopsied 6 days after its transplantation and confirmed, if necessary, by determining the survival time of the test grafts from examination of biopsy specimens taken at regularly spaced intervals. The survival times may then be compared with those relating to grafts on untreated animals.

In the tests with tumour homografts a small piece of viable tumour tissue having the same genetic origin as the injected cells is implanted into the host whose immunity is in question. Then, after a suitable period—which must, of course, be significantly shorter than the *median survival time* (M.S.T.) of tumour homografts in normal hosts—the graft is removed and its viability determined by implanting fragments of it into mice of its own strain of origin.

In all the experiments to be described the recipients were challenged with their test grafts 8–10 days after they had been injected with homologous cells by one route or another.

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Experimental Animals and Control Data

Mice

For the tests in which skin homografts were employed, adult mice belonging to the CBA and A strains were used as donors and recipients respectively. With this combination the M.S.T. of skin homografts transplanted to normal untreated animals is known to be 10·2 ± 0·3 days, and it is also known that graft destruction does not commence until after the 6th post-operative day (Billingham, Brent, Medawar and Sparrow, 1954). Evidence of any destruction in progress in a 6-day biopsy specimen therefore indicates immunity.

For the experiments in which tumour tissue test grafts were used, suspensions of thymocytes were used as the antigenic stimulus. Donors and recipients belonged respectively to the C57 BR/a and A strains. Sarcoma S778, which originated in, and takes uniformly in, C57 BR/a mice was used to challenge the recipients, fragments being implanted subcutaneously by trocar. The biological test for survival of the tumour implants was carried out after they had lain for 8 days in the A-strain hosts. As Table I*b* shows for the controls, 13/14 tumour implants removed after 8 days from normal A-strain mice gave evidence of viability, whereas none of the tumour test grafts removed from 12 animals which had previously reacted against and destroyed homografts of the tumour gave evidence of survival.

Rabbits

Because inbred strains were unavailable, donors and recipients were deliberately selected from different breeds to obtain the maximal degree of genetic disparity—another method of obtaining uniformity of response from different recipients to skin homografts from different donors (Billingham, Krohn and Medawar, 1951). Each rabbit received 3 small pinch grafts of its donor's skin, beds of the appropriate size being prepared in the side of the lateral thoracic wall. These grafts provided biopsy specimens for removal on the 6th, 9th and where necessary 12th post-operative days. Adult males were used throughout, their weights ranging from 1·5–3·5 kg. The median survival time of skin homografts transplanted to rabbits previously immunized with skin grafts from the same donor is 6·0 ± 0·6 days (Medawar, 1944).

Guinea-pigs

The donors were members of a closed pen-inbred colony of albino animals, whereas the recipients belonged to a highly inbred parti-coloured strain, members of which were known to accept skin homografts transplanted between them. Adult males were used throughout, their weights ranging from 500–750 g.

The skin grafting procedures employed have been described in full elsewhere (Billingham and Medawar, 1951).

Preparation of Donor Cells and Their Administration

Suspensions of homologous washed spleen cells, free from undissociated tissue fragments, were used as antigen in all three species. In addition, thymocytes were also used in the mouse, and whole blood, of which the effective element is known to be the leucocytes (Medawar, 1946*a*; Billingham, Brent and Medawar, 1956) was also used in the guinea-pigs.

With mice, the use of inbred strains made preservation of the spleen or thymus donors unnecessary. With rabbits and guinea-pigs, however, the donors had to be kept alive until the time of skin grafting; total splenectomy had therefore to be carried out.

Cell suspensions in Ringer-phosphate solution were prepared from the spleens by the procedure described in full by Billingham and Brent (1957). Suspensions comprising 80–90 per cent thymocytes were prepared by an essentially similar procedure from the organs removed from 6-week-old donors, the medium being Ringer-phosphate solution containing 0·6 per cent glucose. Cell counts were made with a haemocytometer.

With all the injections particular pains were taken to ensure that, so far as possible, the whole of the injection was administered through the intended route. In the event of failure to enter a vein cleanly, the animal was discarded.

Intravenous injections in mice were made in the tail vein, the volume of the inoculum being 0·5 ml. For subcutaneous injection a total volume of 0·5 ml. of suspension was divided equally between the skin of both flanks. Intradermal injections were also made bilaterally, the total volume administered being 0·1 ml. With these injections it was quite certain that most of the material was deposited between the panniculus and the corium rather than

intradermally in the usual sense. For the intramuscular injections the volume of the injection was reduced to 0·03 ml. and it was introduced, *viâ* a no. 30 gauge needle, into the belly of one of the thigh muscles. A small longitudinal skin incision was made beforehand to reveal the muscle. There was no observed tendency for the inoculum to leak out of the muscle on withdrawal of the needle, but this possibility cannot be entirely ruled out.

Guinea-pigs and rabbits received injections ranging from 3–5 ml. Intravenous injection of the guinea-pig was performed through the saphenous vein after its exposure by a small incision just above the "heel". Rabbits were injected through the marginal ear vein. In both species intradermal injections were made bilaterally at a number of scattered sites.

RESULTS

Mice

Tables I*a* and *b* show that with a dose of 5 million cells all 5 routes of administration tested were equally effective in eliciting transplantation immunity; this also held when 4 routes were tested with a dose of 250,000 cells. Five million spleen cells or thymocytes are clearly able to elicit a maximal response. Comparison of the dosages of spleen cells and thymocytes required to achieve complete destruction of the grafts in the majority of subjects suggests that there is little to choose between the antigenic potency of these two cell types. The skin graft test system is, as one would expect, the more sensitive of the two. It revealed that a dose of as little as 2000 spleen cells provoked a just perceptible immunity in 3 out of 5 animals. Whereas the skin graft test system could differentiate between various shades of immunity (by revealing the approximate percentage survival of the graft epidermis), the tumour test system (using the biological

TABLE Ia.—*Percentage of Epithelial Survival in Test Grafts of CBA Skin 6 Days after Transplantation to A-strain Hosts Pre-treated with CBA Strain Spleen Cells by Different Routes*

Number of cells injected	Route of injection				
	Intravenous	Intraperitoneal	Subcutaneous	Intradermal	Intramuscular
5 million	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	25, 5, 0, 0, 0
250,000	50, 25, 0, 0, 0	10, 5, 5, 0, 0	0, 0, 0, 0, 0	—	5, 0, 0, 0, 0
50,000	—	100, 25, 10, 5, 0	—	—	—
10,000	—	75, 50, 25, 25, 10	—	—	—
2,000	—	100, 100, 90, 90, 25	—	—	—

TABLE Ib.—*Percentage of Test Homografts of C57 BR/a Sarcoma Viable on Removal after 8 Days Residence in A Strain Mice Pre-treated with C57 BR/a Thymocytes*

Number of thymocytes injected	Route of injection		
	Intravenous (per cent)	Subcutaneous (per cent)	Intradermal (per cent)
50 million	0 (10)	0 (12)	—
5 ,,	0 (11)	0 (11)	0 (6)
1·58 ,,	0 (9)	17 (12)	—
500,000	36 (11)	40 (10)	—
158,000	100 (5)	70 (10)	—
50,000	83 (12)	82 (11)	100 (6)
5,000	—	100 (11)	—

Proportion of sarcoma grafts surviving on 8th day in A strain hosts immunized with tumour S778 = 0 per cent (12). Proportion of sarcoma grafts surviving in untreated A strain hosts on the 8th day = 93 per cent (14). The figures in brackets are the number of mice tested.

test of survival) merely revealed whether or not *some* viable cells remained in the graft at the time of autopsy.

The results obtained with spleen cells also show that the cell dose response curve is very flat over a wide dose range : from about 2000 to 5 million cells.

Rabbits

The injections of spleen cells into rabbits resulted in immunization by all three routes of administration, but consistently and powerfully only by the intradermal route (see Table II). Intraperitoneal injections gave irregular results in that the reactivity of half the subjects was completely unaltered.

TABLE II.—*Survival Times and Mode of Breakdown in Test Grafts Transplanted to Rabbits Pre-treated with Homologous Spleen Cells by Different Routes*

Number of cells injected (millions)	Route of injection		
	Intravenous	Intraperitoneal	Intradermal
300	<6,* <6*	9, 9, 6*	6*
240	8,* 6*	—	—
160	6,* 8*	6,* 10, 7*	<6,* <6,* 7*

* Indicates that graft breakdown was of the " immune " mode.

Guinea-pigs

The close similarity of the survival times of test grafts transplanted to 4 untreated recipients suggests that a reasonable uniformity of genetic disparity between donors and recipients has been obtained. The survival times of these control grafts are comparable with a M.S.T. of 9·2 days which can be computed from Sparrow's (1953, 1954) data for grafts exchanged between male guinea-pigs from heterogeneous laboratory stocks. With spleen cells it can be seen that all three routes tested were immunologically equivalent (Table III). With leucocytes (in the form of homologous citrated blood), too, there is nothing to choose between the intradermal and intravenous routes. No satisfactory explanation can be offered to account for the one animal which did not become immune after intravenous injection with homologous blood.

TABLE III.—*Survival Times and Mode of Breakdown in Test Grafts Transplanted to Guinea-pigs Pre-treated with Homologous Spleen Cells or Leucocytes by Different Routes*

Type of cell	Number of cells injected (millions)	Route of injection		
		Intravenous	Intraperitoneal	Intradermal
Spleen	90	<6,* <6*	—	6*
	70	—	<6,* 6*	<6*
	60	6*	<6*	<6*
Leucocytes	60	6*	—	—
	55	7*	—	—
	50	<6*	—	6,* <6*
	45	10	—	6*
	25	—	—	6*

Survival times of grafts on controls: 9, 9, 9, 10 days. * Indicates that graft breakdown was of the " immune " mode.

CONCLUSIONS AND DISCUSSION

The results presented here are consistent with the thesis that in mice and guinea-pigs all routes of administration of homologous cells tested are immunologically equivalent and elicit transplantation immunity consistently. However, this equivalence of routes is not found in rabbits. In contradistinction to massive dosages of whole blood, leucocytes or epidermal cells (Medawar, 1946*a* ; Billingham and Sparrow, 1955), spleen cells introduced intravenously in this species do elicit definite though not always powerful transplantation immunity. The intradermal route is here the most effective one, as other workers have found with other types of homologous cell, and the intraperitoneal route gives very irregular results indeed. Billingham and Sparrow (1955) failed to get immunity following two consecutive intraperitoneal injections of epidermal cells, each of which would have been sufficient to elicit transplantation immunity if given intradermally. The inconsistencies obtained with the intraperitoneal route in rabbits may be due to experimental shortcomings rather than to a failure of response on the part of some of the recipients. Stoufer and Paine (1957) have recently investigated the technique of intraperitoneal injection and have found that in the rabbit a surprisingly small proportion of injections are truly intraperitoneal ; many of the inocula find their way into the recipients' intestines even when considerable care is taken to avoid this.

The poor or even non-existent power of rabbit epidermal cells and leucocytes to elicit immunity when injected intravenously contrasts strongly with the powerful immunity which results from the intravenous injection of spleen cells. This anomalous situation may well be explicable in terms of the ultimate fates of these different types of cell. Homologous spleen cells introduced into the blood stream of very young mice are known to settle out electively in lymphoid tissue (Billingham and Brent, 1957), and intraperitoneally injected homologous bone marrow or spleen cells injected into irradiated adult mice show a similar " homing " instinct (Mitchison, 1956 ; Ford, Hamerton, Barnes and Loutit, 1956). The fate of homologous leucocytes or epidermal cells introduced into the blood stream of rabbits may well be different. They may be eliminated from the organism by a mechanism which does not involve any immunological response.

The great disparity between the efficacy of the intradermal and intravenous routes of immunization of rabbits with leucocytes has repeatedly been cited as evidence for the similarity of transplantation immunity and sensitization reactions of the delayed type (Medawar, 1946*b*, 1954 ; Burnet and Fenner, 1949 ; Mitchison, 1954). In the light of the present finding with spleen cells this evidence loses much of its weight.

SUMMARY

The equivalence of different routes of administration of suspensions of homologous cells for the elicitation of transplantation immunity has been compared in the three most common laboratory mammals.

In the mouse, where suspensions of spleen cells or thymocytes were used as the antigen, all five routes tested were equally effective over a wide dosage range. In the guinea-pig, too, in which spleen cells or leucocytes were employed, intravenous, intraperitoneal, or intradermal inoculations were equivalent in their efficacy. With the rabbit, however, spleen cells injected intradermally elicited

a more powerful immune response than when injected intravenously, and intraperitoneal injections gave irregular results. The finding that homologous spleen cells will immunize rabbits when administered intravenously is of interest since both leucocytes and epidermal cells are known to be ineffective when administered by this route.

REFERENCES

BILLINGHAM, R. E. AND BRENT, L.—(1957) *Transplant. Bull.*, **4**, 67.
Iidem AND MEDAWAR, P. B.—(1956) *Phil. Trans. B*, **239**, 357.
Iidem AND SPARROW, E. M.—(1954) *Proc. roy. Soc. B*, **143**, 43.
Idem, KROHN, P. L. AND MEDAWAR, P. B.—(1951) *Brit. med. J.*, i, 1157.
Idem AND MEDAWAR, P. B.—(1951) *J. exp. Biol.*, **28**, 385.
Idem AND SPARROW, E. M.—(1955) *J. Embryol. exp. Morph.*, **3**, 265.
BURNET, F. M. AND FENNER, F.—(1949) 'The Production of Antibodies'. London (Macmillan).
FORD, C. E., HAMERTON, J. L., BARNES, D. W. H. AND LOUTIT, J. F.—(1956) *Nature, Lond.*, **177**, 452.
MEDAWAR, P. B.—(1944) *J. Anat., Lond.*, **78**, 176.—(1946*a*) *Brit. J. exp. Path.*, **27**, 15.—(1946*b*) *Nature, Lond.*, **157**, 161.—(1954) In 'Preservation and Transplantation of Normal Tissues.' Ed. G. E. W. Wolstenholme and M. P. Cameron. London (Churchill).
MITCHISON, N. A.—(1954) *Proc. roy. Soc. B*, **142**, 72.—(1956) *Brit. J. exp. Path.*, **37**, 239.
SPARROW, E. M.—(1953) *J. Endocrin.*, **9**, 101.—(1954) *Ibid.*, **11**, 57.
STOUFER, R. M. AND PAINE, R. H.—(1957) *J. Lab. clin. Med.*, **49**, 313.