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Transplantation Immunity in Annelids
II. Adoptive Transfer of the Xenograft Reaction

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Summary. The oligochaete annelids *Lumbricus terrestris* and *Eisenia foetida* were used to demonstrate adoptive transfer of transplantation immunity. *Eisenia* grafts were used as sensitizing antigen and test grafts. Host *Lumbricus* injected with coelomic fluid containing coelomocytes from *Lumbricus* donors previously sensitized to *Eisenia* grafts rejected test grafts in an accelerated fashion. The rejection time was shorter and significantly different from that of worms injected with saline or coelomocytes from unsensitized worms. Coelomocytes resemble various vertebrate leucocytes and immunocytes and seem equivalent to a hypothetical invertebrate precursor wandering cell which recognizes and reacts to antigen.

INTRODUCTION

Earthworms show evidence of vertebrate-like adaptive immunity characterized by specific rejection of tissue xenografts. Graft destruction is mediated by a memory component; second-set grafts, transplanted 5 days after first-sets, were rejected at a faster rate than the first-sets (Cooper, 1969a). Histological sections seem to indicate that the processes of recognition and rejection are primarily cellular (Cooper, 1968). In this investigation, coelomic cells (coelomocytes) were transferred from putatively immune *Lumbricus* to naive *Lumbricus* to determine their ability to transfer specifically tissue transplantation immunity as revealed by accelerated destruction of test grafts.

MATERIALS AND METHODS

Earthworms, maintenance. First set grafting to Lumbricus *L*1

The worms and techniques have been previously described by Cooper (1968). Donors (L1) and hosts (L2) were grafted with body wall transplants from another oligochaete, *Eisenia foetida*.

*Coelomic fluid from L*1

On the 5th day post-grafting, the coelomic fluid from each *Lumbricus* was removed with disposable pipettes (Aloe Scientific, St Louis, Mo.), previously drawn out over a Fisher burner into micropipettes, attached to a rubber tube with a mouthpiece. The fluid was transferred to separate previously siliconized test tubes (Siliclad, Clay Adams, Inc., New York, N.Y.). Cell counts were made by placing 0·5 ml coelomic fluid and 0·5–1·0 ml

earthworm saline (Rushton's Ringer's solution) in a blood-diluting pipette (Yankee Certified, U.S.A.). One drop of the 1:20 dilution was placed in a Spencer haemocytometer (American Optical Co., Buffalo, N.Y.) and the coelomocytes counted.

Transfer of coelomocytes from L1 (donors) to L2 (hosts)

Coelomocytes were transferred by injecting coelomic fluid anterior and posterior to the graft area at the time of grafting L2 with a 27 G, 1/2-in stainless steel needle (Empire State Thermometer Co., Inc., New York) and 1ml Plastipak disposable syringe (Becton, Dickinson and Co., Rutherford, New Jersey).

Test grafting to Lumbricus *(L2)*

'Second-set *Eisenia* grafts' (actually first-sets on coelomocyte immunized hosts) were performed in the same manner as first grafts, using only one graft from the original donor. Although we have no inbred strains of oligochaetes, the allograft response is slow and our transfer reaction in the L2 hosts occurred faster than would a usual allograft reaction (Cooper, 1969b; Cooper and Rubilotta, 1969).

Antibiotics

Two kinds of antibiotics were used to combat bacterial growth. The antibiotics, Chloromycetin (Chloramphenicol, Parke Davis & Co., Detroit, Michigan) and Streptomycin sulphate powder (Chas. Pfizer & Co., Inc., New York, N.Y.) were sprayed in the graft bed.

Coelomic fluid smears

Preparation of coelomocyte smears was carried out according to routine procedures. Coelomic fluid was smeared on slides and stained with Wright's stain.

Statistics

For statistical analyses of graft survival time we used the 0·05 level as indicating significant difference. The data were analysed by the Mann-Whitney U test (two-tailed) as outlined for non-parametric distributions by Sigel (1956) (Table 2). None of the worms which escaped or died (groups 4 and 5) were included in the calculations.

RESULTS

NORMAL REJECTION OF FIRST-SET XENOGRAFTS ON *Lumbricus* HOST PREVIOUSLY INJECTED WITH RUSHTON'S RINGER'S SOLUTION

These control experiments revealed on two separate occasions that the normal rejections of a first-set *Eisenia* graft on *Lumbricus* is not affected by prior injection of the host with 0·5 ml of Rushton's Ringer's solution. In fifty-six worms the range of beginning rejection of transplants of the two groups was identical, 3–19 days, while the ranges of survival times were 10–88 and 8–69 days. The mean survival times ranged from 25–26 days. We observed no statistical difference between the two groups at the 0·05 probability level. Injecting saline does not affect the host in any way; thus, they do not react differently to subsequent tissue transplants (Table 1, groups 1 and 2).

TABLE 1

SURVIVAL TIME OF *Eisenia* XENOGRAFTS ON UNIMMUNIZED *Lumbricus* (L2) HOSTS WHICH RECEIVED IMMUNE COELOMOCYTES FROM *Lumbricus* (L1)

Group	Type of transfer involving L1 and L2*	Amount of fluid transferred (ml)	No. of grafts	Range in days post-grafting rejection began	Survival time range (days)	Mean survival time (days)	Day of last observation of worms with viable or partially rejected grafts†	Amount of each graft viable (%)	No. of grafts
1	Rushton's Ringer's to L1 (control)	0·5	20	3–19	10–88	26·10	—	—	—
2	Rushton's Ringer's to L1 (control)	0·5	36	3–19	8–69	24·80	—	—	—
3	Non-immune coelomocytes from L1 to L2—No graft to L1 (control)	0·06–0·34	24	3–20	8–52	24·10	—	—	—
4	$6{\cdot}5 \times 10^5$–$2{\cdot}2 \times 10^6$ immune coelomocytes from L1 to L2 (first year)	0·01–0·39	39	2–12	5–43	15·76	19, 24, 33, 119	20, 50, 10, 80	4
5	Immune coelomocytes from L1 to L2 (second year)	0·05–0·54	32	5–16	9–96	21·40	11, 13, 13, 14, 16, 16, 13, 46	100 10, 30	6 2
6	Immune coelomocytes from L1 to L2 (Repeat of second year)	0·10–0·33	23	3–8	6–36	19·65	—	—	—

* Counts of coelomocytes only performed in group 4.
† Escaped or dead hosts.

REJECTION OF XENOGRAFTS ON *Lumbricus* (L2) PREVIOUSLY INJECTED WITH UNSENSITIZED COELOMOCYTES FROM UNGRAFTED *Lumbricus* (L1) GROUP 3

In this experiment involving twenty-four worms, 0·06–0·34ml of coelomic fluid containing coelomocytes from ungrafted *Lumbricus* L1 were injected into host *Lumbricus* (L2) and then grafted. These control results reveal that cells from non-sensitized earthworms were incapable of conferring transplantation immunity to *Eisenia* grafts. The rejection time of 24 days was not significantly different from that of worms receiving saline. This further reveals that without prior sensitization adding more cells to the coelomic cavity was incapable of causing a host to destroy a test graft faster. Thus, cell number is in itself not crucial but the kind of information stored in competent cells is important.

ACCELERATED REJECTION OF FIRST-GRAFTS BY ADOPTIVE TRANSFER (GROUP 4)

During the first several experiments we counted, with great difficulty, the number of cells prior to injection which usually ranged from $6{\cdot}5 \times 10^5$–$2{\cdot}2 \times 10^6$ in 0·01–0·39ml of coelomic fluid. Cell counts were discontinued because of the inability to get a uniform suspension, a problem not yet resolved. In thirty-nine worms, we showed adoptive transfer. Rejection began 2–12 days after transplantation and the survival times ranged from 5 to 43 days. The mean time of ~16 days was significantly different from either of the three control groups. We are unable to explain the minimal number of cells necessary for adoptive transfer, but what is crucial is that the cells were derived from previously sensitized donor earthworms. Within the range of accelerated responses we were able to demonstrate significant differences (Table 2). Only four animals escaped or died with partially viable grafts (Table 1).

TABLE 2

STATISTICAL ANALYSES OF GRAFT SURVIVAL TIMES (DATA FROM TABLE 1)

Groups compared	*P*	
1 with 2	0·76	NS
1 and 2 with 3	0·92	NS
4 with 5	0·032	S
5 with 6	0·16	NS
4 with 6	0·032	S
4 and 5 and 6 with 1 and 2	0·002	S
4 and 5 and 6 with 3	0·0016	S

The 0·05 significance level was accepted as indicating significant population difference. The data were analysed by the Mann-Whitney U (two-tailed) test.

ACCELERATED REJECTION OF FIRST-GRAFTS BY ADOPTIVE TRANSFER (GROUPS 5 AND 6)

In this third experiment we demonstrated again adoptive transfer of the xenograft reaction. In group 5 the mean was slightly greater (21 days) and differed from the previous group 4, but not from group 6 (mean ~20 days) at the 0·05 probability level. Six worms in group 4 escaped or died with well-healed grafts and only two with grafts partially viable.

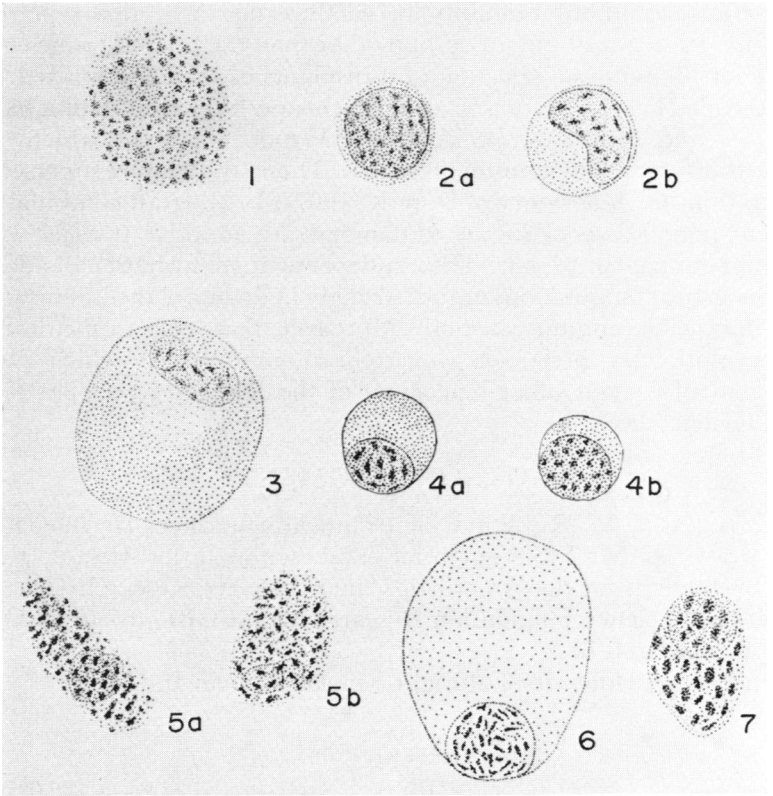

FIG. 1. (1) 'Neutrophil'—light purple cytoplasm; about 14 μ in diameter; nucleus often lobulated and eccentric. (2) 'Lymphocyte'—small cells with basophilic cytoplasm, nucleus central or eccentric. (3) Large 'eosinophil'—about 20 μ, small eccentric oval nucleus; nuclear membrane is absent; cytoplasm contains vacuoles and numerous eosinophilic granules. (4) 'Plasma cell'—variable size from about 8 μ; nucleus eccentric; cytoplasm basophilic. (5) Chloragogen cells—variable in size, no discernible cell membrane; cytoplasm contains numerous round basophilic bodies; small round purple nucleus central or eccentric. (6) Transitional cells—large with eosinophilic granules and basophilic reticulum near nucleus; nucleus eccentric with thin but discernible membrane. (7) Small 'eosinophils'—small cells about 8 μ; distinct cell membrane; nucleus typically single but occasionally bilobed; cytoplasm with large distinct acidophilic granules.

We conclude that the xenograft reaction can be transferred to naive host *Lumbricus* by coelomocytes from a previously sensitized immune host.

COELOMOCYTES IN *Lumbricus*

As can be seen from Fig. 1, the coelomic fluid of *Lumbricus* contains a variety of cells which resemble certain blood cells of vertebrates. The literature reveals variable designations for the cells of the blood and coelomic fluid in earthworms (Stephenson, 1930). In most of our histopathological analyses the cell labelled 3 seems to be the one which predominates in the rejected tissues (Cooper, in preparation).

DISCUSSION

In this paper we described successful adoptive transfer of the xenograft reaction in earthworms. Obviously a refinement of our techniques will yield more information on cell

mediation of transplantation immunity in earthworms. Yet, this represents the first description of adoptive transfer in any group other than the homothermic vertebrates and more evidence for the immune rejection of transplants in an invertebrate by a technique analogous to the classic passive transfer experiments performed by Billingham, Brent and Medawar (1954) and Mitchison (1954, 1955). We do not know which of the cells is responsible for transferring the immune response. These studies were superseded by similar demonstrations from the laboratory of Duprat (1967). In allograft combinations, she used several different populations of *Eisenia* to demonstrate adoptive transfer with quantities (0·05 ml) of fluid equivalent to ours. Thus, independent confirmation of adoptive transfer in allo- and xenogenic combinations argues strongly in favour of the importance of annelid coelomocytes in specific immune reactions. Moreover, these cells fit the description of the hypothetical evolutionary precursor (invertebrate mobile cell which recognizes and reacts to antigen) of various other leucocytes of the RES and vertebrate immunocytes suggested by Burnet (1968).

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