Transfer Factor: A Subcellular Component that Transmits Information for Specific Immune Responses

(DELAYED HYPERSENSITIVITY IMMUNE RESPONSE/LEUKOCYTE EXTRACTS/TRANSFER OF IMMUNOLOGICAL INFORMATION/GUINEA PIG)

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ABSTRACT Two transfer factors have been prepared in an animal model system, the guinea pig. They are both small molecules, derived from the leukocytes of immunologically experienced individuals, and capable of transferring information for specific immune responses to naive individuals.

The molecular events involved in the development of an immune response represent a largely unsolved problem. Numerous steps intervene between the introduction of antigen and the mounting of the full immune response. These steps often include the production of macromolecular mediators, which serve as signals between the participating cells.

Transfer factors are a controversial example of molecules that mediate immunological information. They are subcellular components, derived from the leukocytes of an educated individual, that can be used to transmit immunological information to a naive individual.

The transfer factor phenomenon arose in 1954 from the work of Lawrence with human beings (3) and of Jeter, Tremaine, and Seebohm with guinea pigs (4). Their experiments began with individuals who were exposed to antigens such as streptococcal M protein or dinitrochlorobenzene. This exposure provoked a primary immune response that not only eliminated the foreign agent but also established a long-lived memory within the immune system. Thus, when the animals were exposed again to antigen, they mounted a secondary response that was both stronger and more rapid than the primary response.

The key observation of Lawrence, and of Jeter, Tremaine, and Seebohm, was that extracts prepared from the leukocytes of the sensitized individuals contained a substance that could transmit immunological memory (or preparedness) to a naive individual. Thus, the naive individual, upon receipt of the leukocyte extract, could immediately mount a secondary immune response upon its first exposure to the antigen.

In subsequent years the transfer factor phenomenon was extended to include several other immune reactions; for instance, responses against transplantation antigens (5), bacterial spores (6, 7), and such foreign proteins as tuberculin (8), diphtheria toxin (9), and modified serum components (10). All of these immune responses have in common the property that they are "cell-mediated." That is, they can be directly carried out by intact leukocytes, as opposed to leukocytes that function indirectly through the secretion of freely circulating antibodies. Whether or not there are similar transfer factors associated with humoral immune responses remains unknown.

Transfer factors are of interest, not only because of their promise in helping to understand the mechanisms involved in the immune response, but also because of their potential application in the treatment of diseases. For instance, transfer factors have been used with encouraging results in a variety of immunodeficiency diseases (11), infections (7), and neoplasias (12).

The physical identity of the transfer factors has been a problem of long-standing interest. Lawrence has shown that human transfer factor is resistant to DNase, pancreatic RNase, and trypsin (13). He has also made the important observation that the transfer factors are small enough to pass through a dialysis membrane (14). This finding poses an interesting problem, for it means that the transfer factors are too small either to be or to code for the proteins involved in a specific immune response. A regulatory role for transfer factor appears to be indicated.

We have studied the transfer factor phenomenon in guinea pigs, drawing on the procedures introduced by Jeter, Burger, Tremaine, and Seebohm (4, 15) and by Guthrie, Ellis, and Brock (16). The general purpose of the experiments is 2-fold: to further characterize the reaction mediated by transfer factor, and to attempt to inactivate the transfer factor enzymatically and, thereby, determine its chemical nature.

As discussed in this paper, we have been able to confirm the transfer factor phenomenon (which has been a matter of considerable dispute; see, for instance, ref. 17). We have observed the transfer factor phenomenon in 30% of our preparations, and often at a 90-fold higher efficiency than previously reported. The donor:recipient ratio has been 1:15 rather than 6:1. Two types of transfer factor have been prepared. One primes an animal to respond to dinitrochlorobenzene (DNCB), and the other primes the recipient to respond to ortho-chlorobenzoylchloride (OCBC). The results obtained with these preparations support the conclusion that transfer factors program specific immune responses, as opposed to functioning as general immune system activators.

As described in the following paper (18), we have carried out an enzymological analysis that indicates that the biological activity of these transfer factors resides partly or
entirely in species of low molecular weight, double-stranded RNA.

**Sensitization of animals that will donate transfer factor**

The experimental system we use is the guinea pig, and the immune response we have studied is the reaction to small chemical hapten. The intent is to sensitize animals to either DNCB (1-chloro-2,4 dinitrobenzene) or OCBC (ortho-chloro-benzoylchloride), and later to use their lymphoid tissue as a source of transfer factor. The animals used were randomly bred, Hartley strain males, obtained from the Charles River Breeding Farms of Wilmington, Mass.

Animals were sensitized by pipetting 5 μl of a 50% DNBC or OCBC solution (w/v in acetone) onto the dorsal surface of one ear. The DNBC or OCBC permeates the highly vascular tissue of the ear and, eliminating its reactive chlorine, becomes conjugated to the ε-amino groups of lysines and to the sulf-hydryl groups of cysteines, causing numerous proteins to appear foreign. During the following week the animals mount a primary immune response and establish immunological memory. Thereafter, if the antigen is applied again, the animals should give a secondary response, which in this case is called a delayed hypersensitivity response. This term for the secondary response denotes that the animal is hypersensitive to the antigen and responds maximally after a slight delay (18–24 hr).

The delayed hypersensitivity response is characterized by several stages of increasing severity. We score the reactions as in Chart 1.

<table>
<thead>
<tr>
<th>Response</th>
<th>Symptom</th>
<th>Cause</th>
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<tbody>
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<tr>
<td>0</td>
<td></td>
<td></td>
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<tr>
<td>+1</td>
<td>Patchy erythema</td>
<td>Dilation of blood vessels in the exposed region, causing an increased circulation of blood</td>
</tr>
<tr>
<td>+2</td>
<td>Homogeneous erythema</td>
<td></td>
</tr>
<tr>
<td>+3</td>
<td>Edema and induration</td>
<td>Further vasodilation leading to the leakage of plasma fluid and leukocytes into the affected area</td>
</tr>
<tr>
<td>+4</td>
<td>Patchy necrosis</td>
<td>Generalized tissue destruction caused by scavenger macrophages elicited by the specific responding leukocytes</td>
</tr>
<tr>
<td>+5</td>
<td>Homogeneous necrosis</td>
<td></td>
</tr>
</tbody>
</table>

controls in every assay for sensitization, never gave a response to challenge with antigen.

The skin reaction was invariably specific for the antigen involved: animals sensitized to DNBC responded to DNBC but did not give a delayed hypersensitivity response when simultaneously challenged with OCBC. The converse of this statement was also true: animals sensitized to OCBC responded only to challenge with OCBC, not to challenge with DNBC.

**Preparation of the transfer factors**

The DNBC- and OCBC-sensitized animals were used as the source of transfer factor. For a standard preparation, three animals were processed. On day 7, each animal was injected intraperitoneally with 25 ml of heavy paraffin oil. This was done in order to induce a nonspecific irritation of the peritoneal cavity (peritonitis) which would result in an increased concentration of leukocytes in the abdominal cavity.

**Figs. 1-5 (on preceding page).**

In this paper we study, in guinea pigs, the phenomenon of transfer factor—that is, the transmission of specific immunological information from a population of “educated” leukocytes to a population of “naïve” leukocytes. The figures show the type of immune responsiveness that is present in the sensitized donors and that can be transmitted to naïve recipients by transfer factor.

**Fig. 1.** Immune capacity developed in an animal that has been directly exposed to antigen (DNBC or OCBC). Six days after antigen was painted on the ear, the back of the guinea pig was shaved and the antigen was applied again. This provoked the secondary response (a delayed hypersensitivity reaction) shown. The reaction is designated +2 in severity; it is characterized by a homogeneous erythema (redness) which represents an increased blood supply in the area where responding leukocytes are eliminating the antigen.

**Fig. 2.** A close-up picture of the reaction site (A) compared to an adjacent area not exposed to antigen (B). The lymphoid tissue of animals such as the one shown in Figs. 1 and 2 serves as the source of transfer factor. The transfer factor is a subcellular material derived from this tissue.

**Fig. 3.** Acquired immune capacity of two naïve animals that received injections of transfer factor, and the reactivity of a third animal that was mock injected with a saline solution. Upon challenge with DNBC (which here is the first exposure to antigen) the two test animals both mounted secondary responses. The animal on the left, which received the transfer factor from one-fourth of a donor, was able to produce very strong delayed hypersensitivity responses upon challenge with antigen. The reactions are scored as +5, being characterized by homogenous necrosis of the affected region, in addition to erythema and induration (swelling). The middle animal received transfer factor from one-eighth of a donor and was able to give a +4 response (patchy necrosis) to the higher test solution of antigen used, and a +3 response (erythema and induration) to the lower test solution of antigen. (The slight erythema on the upper left flanks of the test animals was in response to a very low concentration of antigen, 5 mM DNBC.) The control animal on the right, which received saline solution instead of transfer factor, was not able to give a response upon challenge with antigen.

**Fig. 4.** Data concerning the specificity of the immune responsiveness transmitted by transfer factor. This animal has been injected with transfer factor prepared from donors that had been sensitized to give a delayed hypersensitivity response to OCBC. The animal that received the transfer factor was then challenged with both DNBC and OCBC; he responded only to OCBC (upper flank). The response is +3 in severity (erythema and induration).

**Fig. 5.** The swelling component of the reaction in Fig. 4 is particularly well displayed here, where the reaction site has been pinched.
On day 11, the animals were sacrificed, using ether and cervical dislocation. A small incision, about 2 cm in diameter, was then made in the abdominal wall and 50 ml of Hank's balanced salt solution was introduced into the peritoneal cavity. With intermittent massaging of the abdominal walls to distribute the fluid, the suspension of oil and Hank's solution was pipetted into centrifuge tubes on ice. In this way leukocytes were washed off the peritoneal wall and the surrounding viscera. The process was repeated with a second 50-ml portion of Hank's solution. The suspension of peritoneal exudate cells was spun at 1500 rpm in the Sorvall HB-4 rotor for 10 min at 4°. The cell pellet was resuspended in 5 ml of Hank's solution and kept on ice.

The abdominal incision was then enlarged and the spleen was removed and placed on ice in a petri dish containing Hank's solution. The spleen was cut into about 10 pieces and homogenized with a Dounce homogenizer, using 5 ml of Hank's solution. The insoluble fibrous residue was allowed to settle and was discarded.

Finally, the lungs were removed. They were placed in a petri dish on ice with 5 ml of Hank's solution, and minced with surgical scissors. Alveolar leukocytes were washed free by gently squeezing the minced tissue in the buffer. The remainder of the lung tissue was discarded.

The peritoneal cell suspension, the spleen homogeneate, and the alveolar wash were pooled. The cells were then centrifuged as described above and resuspended in 7.5 ml of Hank's solution. They were then incubated at 37° for 4.5 hr on a roller drum, a procedure introduced by Guthrie, Ellis, and Brock (16). After 1 hr of incubation a cell count was made, showing a density of about 10^9 cells per ml. The majority of the cells were leukocytes.

After 4.5 hr, the cell suspension was again centrifuged, as described above. The supernatant was collected and dialyzed for 20 hr against 200 ml of 150 mM NaCl and 5 mM Tris buffer, pH 8. The dialysate was lyophilized to dryness and weighed, giving a final mass of about 2.0 g from the original three guinea pigs. About 1.8 g of this mass could be attributed to the NaCl in the dialysis buffer.

The preparation was dissolved in 5 ml of distilled water and stored frozen.

**Assay of the transfer factor preparations**

The lyophilized dialysates were tested to see if they contained transfer factor activity—that is, if they could transfer to animals that had never been exposed to DNCB or OCBC the ability to give a delayed hypersensitivity response upon first exposure to the antigen.

Fig. 3 shows results that were typical for the transfer factor preparations. Three naive animals were injected intraperitoneally: Animals on the left and in the middle received 0.5 ml and 0.25 ml of DNCB transfer-factor preparation, respectively; the control animal (on the right) received 0.5 ml of 3 M NaCl. The use of 0.25 ml of the transfer factor preparation represented an attempt to sensitize a recipient with the material from about 15% of an original sensitized donor. Forty-eight hours after injection, the animals were shaved and challenged on the upper flank with 35 μl of 50 mM DNCB in acetone-olive oil. A second challenge site on the lower flank received a lower concentration of DNCB (25 mM).

Responses began to appear 10–14 hr after challenge. It was clearly evident by 18 hr that the animals that had received transfer factor had developed pronounced +3 responses, characterized by erythema (redness) and induration (swelling). By 36 hr, the guinea pig that had received 0.5 ml of transfer factor (left) showed +5 reactions (homogeneous necrosis) to both the higher and lower test solutions of DNCB. The animal that had received 0.25 ml of transfer factor (middle) showed a +4 response to the higher concentration of DNCB and a +3 response to the lower DNCB concentration. Because of the time course of appearance of these responses, we have accepted them to be delayed hypersensitivity reactions.

The naive control animal (right), which had been injected with 0.5 ml of a 3 M NaCl solution rather than transfer factor gave no response whatsoever to challenge with DNCB. Thus, the concentrations of DNCB used for challenging, and the procedures involved, were not in themselves irritating.

The delayed hypersensitivity responses mediated by the OCBC transfer factor preparations were identical to those described above.

So far, we have made 40 DNCB preparations, 14 of which were successful; and 15 OCBC preparations, five of which were successful. We are exploring other procedures for preparing transfer factor because it is the nonreproducibility of the current methods that poses the most serious block to further progress. Nonetheless, it should be emphasized that once an active preparation has been obtained, it will function in every recipient. This statement is based on the transfer of delayed hypersensitivity capacity to about 150 naive animals.

**Specificity of the responses mediated by transfer factors**

Do transfer factors program specific immune responses, or are they involved in a generalized priming of the immune system for response to any antigen? Fig. 4 presents data indicating that the transfer factors transmit information for specific immune reactions.

Two naive animals were injected, one with OCBC transfer factor and the other with DNCB transfer factor (not shown). Forty-eight hours later both animals were challenged simultaneously with 35 μl of 700 mM OCBC (on the upper flank) and 50 mM DNCB (on the lower flank). The animal injected with OCBC transfer factor responded only to challenge with OCBC and gave no response to DNCB; conversely, the guinea pig that received DNCB transfer factor mounted a full response to challenge with DNCB but did not respond to OCBC. Fig. 4 shows the response after 18 hr of the animal that received OCBC transfer factor. Fig. 5, in which the reaction site has been pinched, is designed to show the swelling component of this strong +3 response.

Specificity experiments such as the one above have been performed on four independent DNCB and OCBC transfer factor preparations.

**Summary**

As first described by Lawrence (1, 2) and by Jeter, Tremaine, and Seebohm (4), transfer factors are small dialyzable components that can be extracted from the leukocytes of a sensitized individual and used to transmit immunological information to the leukocytes of a naive individual. Specifically, transfer factors transmit immunological memory and allow an animal that has never been directly exposed to an antigen to mount a secondary immune response upon challenge.

Our results are in full accord with the transfer factor phenomenon. Using the guinea pig as an experimental system, and the delayed hypersensitivity reaction as an immune response, we have prepared transfer factor from animals sensi-
ized to two antigens. These are dinitrochlorobenzene and ortho-chlorobenzoylchloride. The efficiency of these transfer factor preparations is often ninety-fold higher than previously reported, allowing the material from one donor to sensitize ten to fifteen recipients.

Both transfer factor preparations behave as if they transfer the information for a specific immune response rather than activating the immune system in a general sense. Thus, animals that received DNCB transfer factor respond to challenge with DNCB, but not to challenge with OCBC, and vice versa.

How certain can we be that the transfer factor phenomenon is real? Throughout its history, transfer factor has been a matter of considerable dispute; a critical review of the developing data can be found in ref. 17. We believe that we have seen the transfer factor phenomenon in at least 20 independent preparations. The reactions observed were pronounced, and exactly as shown in Figs. 3-5. On the four occasions when we simultaneously tested DNCB and OCBC transfer factor preparations for the specificity of the immunological information they transmitted to naive individuals, the results clearly indicated specificity. All of the steps in the preparation and testing of transfer factor have been done both collaboratively and independently by each of the present authors and by Huntington Potter, who has joined our research group. Our major problem is the fact that, at least in our hands, the preparation of transfer factor is a matter of chance, occurring with a success rate of 30%. This clearly means that the isolation of biologically active transfer factor depends on variables that are not yet understood and are therefore not adequately described in the procedures section. Our current efforts are largely directed toward finding a solution to this problem.

Using these transfer factor preparations, we have performed an enzymological analysis that indicates that the biological activity of transfer factor is contained partly or entirely in low molecular weight, double-stranded RNA molecules. Also, we have been able to measure the size of the transfer factors on polyacrylamide gels. These results will be presented in two succeeding papers (18; H. Potter and D. Dressler, manuscript in preparation), and in considering them we will discuss some of the possible ways in which transfer factors might function as carriers of immunological information.

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