

Influence of Cellular Factors on Immune Unresponsiveness Induced by *Klebsiella pneumoniae* Capsular Antigen

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The mechanisms by which immune unresponsiveness (immune paralysis) develops are still uncertain. The present work was based on the assumption that this condition may be due to failure of certain activities of the macrophages. Data from passive transfer of such cells are interpreted as supporting this hypothesis. Purified capsular polysaccharide from type 2 *Klebsiella pneumoniae* induced immunity when given to Swiss albino mice in 5- μ g amounts and immune paralysis when given in 1,000- μ g amounts. The unresponsive state lapsed to that of acquired immunity 3 months after induction. Passive transfer of cells from groups of *K. pneumoniae* immune, "paralyzed," and control mice showed that peritoneal cells from "paralyzed" donors induced significant protection against challenge in both control and "paralyzed" recipients. In contrast, spleen cells from control, "paralyzed," and immune animals failed to effect such transfer. The finding that unresponsiveness could be terminated by peritoneal cells and not spleen cells indicates that macrophages played a primary role in immune paralysis, possibly owing to their loss of capacity to transfer factors or information for induction of antibody synthesis.

The term immune unresponsiveness ("paralysis") is used to denote a condition in animals given excessively large amounts of certain antigens. The result of such treatment is the inability of the animal to produce specific antibody while displaying full susceptibility to pathogens possessing the antigens involved. Most infection susceptibility studies have been made with mice and *Diplococcus pneumoniae* (6, 16) or *Klebsiella pneumoniae* (2) as the experimental model.

There is at present a lack of convincing direct evidence regarding the mechanism of this type of unresponsiveness. The few studies done have led to conflicting explanations. One hypothesis is that excess antigen binds the animal's antibody such that the latter cannot exert its protective effect on challenge (5, 21). Another is that excess antigen itself suppresses production of specific antibody-forming cells or destroys them in some unknown manner (4, 16, 19).

Cellular immunity, the enhancement of phagocytic activity after exposure to antigen, is known to be involved in actively acquired immunity in many types of infections (e.g., 12, 14). Furthermore, macrophages may be involved in the pre-

liminary processing of antigen as a requisite for antibody-producing cells subsequently to carry out their function (7, 20).

This study sought to bring together the concepts involved in immune paralysis and in cellular immunity. Indeed, as a working hypothesis, macrophages were considered to be intimately involved in "paralysis," and the incapacitation of the immune system occurred at the cellular level. Evidence was obtained to substantiate this point of view.

MATERIALS AND METHODS

Organism. A *K. pneumoniae* culture originally obtained from the University of California Medical Center, San Francisco, was employed in these studies. It was found to be serotype 2 by Quellung reaction through use of commercial antiserum (Difco). Stock cultures were maintained in Worfel-Ferguson Broth (Difco) at room temperature or frozen at -40 C. Mouse passage was performed approximately every 2 weeks to maintain virulence of the organism.

Numerous virulence determinations showed that the organism varied relatively widely in its virulence characteristics; an LD₅₀ dose ranged from 8 to 160 organisms. Animal challenges were made employing 10 to 50 LD₅₀ doses, 1 LD₅₀ being represented by that given in the latest virulence determination prior to challenge in question. Despite variability in the or-

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ganism's virulence, it does not detract from overall data in view of the uniformity of results.

Animals. Commercially obtained 20- to 25-g Swiss albino female mice (Berkeley-Pacific Laboratories, Berkeley, Calif.) were employed in all the experiments to be described. Animals were maintained with commercial food pellets and water.

Isolation of *K. pneumoniae* purified capsular polysaccharide. Capsular polysaccharide of type 2 *K. pneumoniae* was isolated by a modified method of Heidelberg, Kendall, and Scherp (9). Approximately 12 liters of the organism were grown in Worfel-Ferguson Broth at 37 C on a reciprocating shaker. After 3 days, Formalin was added to a final concentration of 0.3% to kill the organisms. Next, cultures were concentrated to approximately 0.1 the original volume by pervaporation. Cells were then removed by centrifugation at 10,000 rev/min for 10 min at 5 C. Two volumes of 95% ethyl alcohol were added to the stirred supernatant fluid, and the preparation was allowed to remain in the refrigerator overnight to precipitate capsular polysaccharide. The latter was collected by centrifugation, redissolved in 250 ml of distilled water containing 10 g of sodium acetate, and was dialyzed against distilled water for 24 hr at 4 C. The material from the dialysis tubing was subsequently deproteinized by agitation with equal volumes of 1:5 *n*-butanol-chloroform mixture, followed by centrifugation to remove precipitated protein. Six such cycles were required to give an adequate preparation. Finally, the polysaccharide was again dialyzed against distilled water, reprecipitated with two volumes of 95% ethyl alcohol, and centrifuged to collect polysaccharide. The final product was dried over phosphorus pentoxide in a vacuum desiccator.

The amount of nitrogen in the purified polysaccharide was determined by the titrimetric micro-Kjeldahl procedure of Hiller, Plazin, and Van Slyke (10). By this method, it was determined that 1.23% nitrogen was present. This is comparable to that described for the BB preparation used by Batshon, Baer, and Shaffer (2).

Establishment of immunizing and "paralyzing" polysaccharide dosages. Immunizing and "paralyzing" polysaccharide dosages were established by first dissolving purified *K. pneumoniae* polysaccharide in pyrogen-free saline (PFS; Don Baxter, Inc., Glendale, Calif.) at a concentration of 1,000 $\mu\text{g}/\text{ml}$. After filter sterilization, the solution was diluted to desired concentrations with PFS.

Groups of mice were injected intraperitoneally (ip) with various concentrations of antigen, along with a control group which received an equivalent volume of PFS only. All animals were challenged 14 days after treatment with 10 or 50 LD₅₀ doses of a 24-hr culture of *K. pneumoniae*, and mortality was recorded for 14 days.

Passive transfer of peritoneal and spleen cells. Passive cell transfer experiments were accomplished to ascertain effects of peritoneal or spleen cells from control, immune, or unresponsive donors on similarly prepared groups of recipients. Donor peritoneal cells were obtained from each of the three treated groups of mice 14 days after PFS or antigen injection. This

was accomplished by injecting the animals ip with 5 ml of NCTC 109 medium containing 1 unit of heparin per ml (Nutritional Biochemicals Corp., Cleveland, Ohio). They were anesthetized and were bled by intracardial puncture, and then the peritoneal cavity was aseptically exposed. An 18-gauge aspirating needle and a 5-ml syringe were used to aspirate gently the injected fluid and the peritoneal cells. The latter were pooled within groups of animals and were held in an ice bath. Next, spleens were removed and pooled within groups in ice-cooled NCTC 109 medium. Spleen cells were released in Ten Broeck tissue grinders.

Suspensions of pooled macrophage or spleen cells from each group of mice were sedimented in the cold by centrifugation at $350 \times g$; the supernatant fluid was discarded, and cells were resuspended in chilled NCTC 109 medium. Mononucleated cells in each pool were enumerated, and cell viability was determined with eosin Y, the nonviable cells absorbing the dye. Finally, peritoneal cells were diluted to a concentration of 9.5×10^6 and spleen cells to 200×10^6 cells/ml before injection into recipient mice.

Control, immune, or unresponsive recipients which had been obtained by administration of PFS or antigen 14 days earlier were given either 9.5×10^6 peritoneal cells or 200×10^6 spleen cells ip. Experimental controls included similar groups (control, immune, or "paralyzed") which received 1 ml of NCTC 109 medium only. All animals were challenged 24 hr after cell transfer with 50 LD₅₀ doses of *K. pneumoniae*, and mortality was recorded over 14 days.

Additional experiments were carried out to determine the effects of disrupted peritoneal or spleen cells. The procedure was virtually the same as described above for viable cells, except 9.5×10^6 peritoneal cells or 200×10^6 spleen cells per ml from control, immune, or "paralyzed" animals were exposed to alternate freezing in a dry ice-acetone bath and thawing at 37 C for five cycles. Microscopic examination revealed no intact cells after such treatment. A 1-ml amount of disrupted cells was then injected ip into recipient groups of mice as previously described.

Antibody assays. *K. pneumoniae* antibody titers were determined from serum pools of animals receiving no purified *K. pneumoniae* polysaccharide, or of those receiving immunizing and "paralyzing" dosages. The assay was performed by Quellung reaction.

Only those receiving immunizing dosages responded with detectable antibody formation, titers being approximately 1:32.

RESULTS

Assay of immunizing or immunologically paralyzing effect of purified *Klebsiella pneumoniae* capsular polysaccharide. *K. pneumoniae* capsular polysaccharide was prepared as described in the Materials and Methods section. Antigen concentrations of 5, 50, 500, 750 and 1,000 $\mu\text{g}/\text{ml}$ were administered ip to groups of 10 Swiss albino mice, 20 to 25 g each. An additional control group received only PFS. The assay was replicated five times in the groups receiving 50 to 1,000 μg ,

giving data for a total of 50 mice in each of these, and seven times in the control and groups receiving 5 μg , giving a total of 70 animals for this assay. All animals were challenged with 10 LD_{50} doses (50 to 1,600 organisms, depending on results of prior LD_{50} determination) of a 24-hr culture of *K. pneumoniae* 14 days after administration of antigen or PFS. Table 1 indicates the actual number and the per cent mortality found from these tests.

It is evident from Table 1 that *K. pneumoniae* polysaccharide evoked a definite immunizing and unresponsive effect. A 5- μg amount of polysaccharide produced the lowest mortality rate. With increasing dosages, mortality increased. At 750 μg , the level of resistance had been reduced to that of controls, and such animals were immunologically unresponsive. However, 1,000 μg of polysaccharide produced resistance even lower than that in the control animals. Other workers have reported that similarly treated animals are immunologically paralyzed and fail to become resistant on exposure to an optimal antigenic dose (6, 17). All subsequent experiments described here in which large dosages of antigen did not lead to increased resistance are defined as a demonstration of this condition.

Based on these data, 5 μg was selected as an immunizing dose of *K. pneumoniae* polysaccharide and 1,000 μg as an appropriate "paralyzing" amount.

Duration of immune paralysis in mice due to K. pneumoniae capsular polysaccharide. The duration of "paralysis" has been controversial. On one hand, it has been reported to be permanent for *D. pneumoniae* capsular polysaccharide in mice (6), whereas a finite duration has been demonstrated for immune paralysis induced by *K. pneumoniae* polysaccharide (2). Accordingly, 40 mice were "paralyzed" with 1,000 μg of antigen

TABLE 1. Mouse response to varying *Klebsiella pneumoniae* purified polysaccharide dosages^a

| Polysaccharide dose (μg) | Total mortality (dead/total) ^b | Avg per cent mortality |
|---------------------------------------|---|------------------------|
| 0 ^c | 31/70 | 44.0 |
| 5 | 6/70 | 8.6 |
| 50 | 9/50 | 18.0 |
| 500 | 12/50 | 20.0 |
| 750 | 24/50 | 40.0 |
| 1,000 | 25/50 | 50.0 |

^a All animals were challenged 14 days after polysaccharide or pyrogen-free saline administration, and mortality was observed for 14 days.

^b Cumulated total of five or seven replicate experiments by use of 10 animals per group.

^c Control group received PFS only.

and were challenged at monthly intervals with 50 LD_{50} doses (5,000 organisms) of a 24-hr culture of *K. pneumoniae* to determine the duration of effect with our product. The data are summarized in Table 2.

It is evident that the "paralyzed" state in mice was maintained for approximately 3 months. Actually, a slight decrease in susceptibility was noted by this time. Therefore, it appears that for this product "paralysis" is of limited duration.

Passive transfer of peritoneal and spleen cells. Several experiments were performed to elucidate the role of cellular factors in *K. pneumoniae* infection of mice, based upon the assumption that passive transfer of macrophages from immune mice would provide protection for control animals, or perhaps even for "paralyzed" animals. Thus, *K. pneumoniae* capsular polysaccharide or PFS was given to provide immunized, "paralyzed," and control groups.

After harvesting, washing, enumeration, and testing of viability, 9.5×10^6 peritoneal cells or 200×10^6 spleen cells were transferred to recipients. The latter were challenged 24 hr later with 50 LD_{50} doses (5,000 organisms) of *K. pneumoniae*. Additional groups of control, immune, and "paralyzed" animals receiving no cells were included as controls.

Donor cell viability was greater than 90% in all cases. It appeared that both peritoneal and spleen cells from "paralyzed" mice had a small but uniformly increased susceptibility to effects of harvesting and washing over those from control and immune animals.

The combined data of passive peritoneal cell transfers is shown in Table 3. Highly significant protection was provided control and "paralyzed" recipients that were given peritoneal cells from "paralyzed" donors as compared to control or "paralyzed" recipients receiving cells from control animals [$P = 0.007$ and 0.034 , respectively, when computed by the method of Fisher and Irwin (11)]. "Immune" cells did not offer protection to

TABLE 2. Duration of immune paralysis in mice treated with 1,000 μg of *Klebsiella pneumoniae* purified polysaccharide

| Elapsed time after administration of polysaccharide (months) | Mortality (dead/total) ^a |
|--|-------------------------------------|
| 1 | 9/10 |
| 2 | 9/10 |
| 3 | 7/10 |
| 4 | 1/10 |

^a Challenged with 50 LD_{50} doses of *K. pneumoniae*.

TABLE 3. Mortality rates of control, immune, and "paralyzed" mice receiving 9.5 million peritoneal cells from control, immune, and "paralyzed" donor mice^a

| Recipient group | Source of donor cells | Mortality (dead/total) ^b | Per cent mortality | P values ^c |
|-----------------|-----------------------|-------------------------------------|--------------------|-----------------------|
| Control | None ^d | 13/15 | 52 | 0.500 0.007 |
| | Control | 8/15 | 53 | |
| | Immune | 7/15 | 47 | |
| | "Paralyzed" | 2/20 | 10 | |
| Immune | None | 1/20 | 5 | 0.500 0.728 |
| | Control | 2/15 | 13 | |
| | Immune | 1/15 | 7 | |
| | "Paralyzed" | 3/20 | 15 | |
| "Paralyzed" | None | 15/20 | 75 | 0.786 0.034 |
| | Control | 10/15 | 67 | |
| | Immune | 11/15 | 73 | |
| | "Paralyzed" | 6/20 | 30 | |

^a Control donors and recipients were given 1 ml of PFS; immune groups, 5 µg, and "paralyzed" groups, 1,000 µg of *K. pneumoniae* polysaccharide in 1 ml of PFS ip 14 days prior to cell transfer.

^b Challenged ip with 50 LD₅₀ doses of *K. pneumoniae* 24 hr after cell transfer.

^c Computed by method of Fisher and Irvin (see 11). All values were based upon cell transfer controls within each group (i.e., "paralyzed" recipients receiving "paralyzed" cells compared to "paralyzed" recipients receiving cells from control animals, etc.).

^d Challenge controls not given passive transfer of cells.

any group of recipient mice showing inability to transfer cellular immunity. As expected, immune recipients were not significantly affected by transfer of any donor cells, thus reflecting their high degree of resistance due to immunization.

Table 4 provides mortality rates and statistical values for mice receiving spleen cells from various donors (combined data from two experiments). Spleen cells from "paralyzed" or control groups did not offer significant protection to "paralyzed" or control recipient mice. A comparison of "paralyzed" recipients receiving "immune" cells with those receiving "paralyzed" cells, however, indicates the former were provided a significant degree of protection.

Passive transfer of disrupted peritoneal and spleen cells. Since intact peritoneal cells from "paralyzed" hosts induced resistance to control and "paralyzed" recipients, it was important to determine whether this depended on viable cells. Accordingly, peritoneal and spleen cells were obtained from the variously treated groups of

mice as previously described. After washing, enumeration, and viability determination, cell suspensions were adjusted to that employed in transfer of intact cells (9.5×10^6 peritoneal cells and 200×10^6 spleen cells/ml). The cells were then disrupted by alternate freezing at -70°C and thawing at 37°C . Of each cell residue, 1 ml was then injected into groups of control, immune, or "paralyzed" recipients.

Results for transfer of disrupted macrophages of disrupted spleen cells are shown in Table 5.

TABLE 4. Mortality rates of control, immune, and "paralyzed" mice receiving 200 million spleen cells from control, immune, or "paralyzed" mice^a

| Recipient group | Source of donor cells | Mortality (dead/total) | Per cent mortality | P values |
|-----------------|-----------------------|------------------------|--------------------|----------------|
| Control | None | 13/25 | 52 | 0.500 0.624 |
| | Control | 10/20 | 50 | |
| | Immune | 11/20 | 55 | |
| | "Paralyzed" | 10/20 | 50 | |
| Immune | None | 1/20 | 5 | 0.043 0.105 |
| | "Paralyzed" | 0/10 | 0 | |
| "Paralyzed" | None | 15/20 | 75 | 0.043 0.105 |
| | Control | 10/10 | 100 | |
| | Immune | 6/10 | 60 | |
| | "Paralyzed" | 7/10 | 70 | |

^a Refer to footnotes of Table 3.

TABLE 5. Effect of disrupted peritoneal and spleen cells from control, "immune," and "paralyzed" donor mice on control and "paralyzed" recipient mice^a

| Recipient group | Source of donor cells ^b | Per cent mortality | |
|-----------------|------------------------------------|----------------------------|------------------------|
| | | Peritoneal cell recipients | Spleen cell recipients |
| Control | None | 80 | 78 |
| | Control | 100 | 90 |
| | Immune | 100 | 100 |
| | "Paralyzed" | 60 | 80 |
| "Paralyzed" | None | 60 | 60 |
| | Control | 100 | 97 |
| | Immune | 100 | 95 |
| | "Paralyzed" | 60 | 80 |

^a Refer to Table 3 footnotes.

^b Alternate freezing and thawing was employed to disrupt 9.5×10^6 donor peritoneal cells per ml or 200×10^6 spleen cells per ml.

Disrupted "immune" peritoneal cells did not increase the resistance of recipient "paralyzed" mice, whereas those from "paralyzed" donors appear to offer some protection to both control and "paralyzed" recipients (60% mortality of recipients of "paralyzed" cells versus 100% mortality of those receiving cells from control mice). Statistical computations reveal that these differences are not significant. Similarly, data of Table 5 reveal that in no case was protection afforded to the recipients by transfer of disrupted spleen cells.

DISCUSSION

The overall objectives of this work were to (i) assess the role of cellular immunity in *K. pneumoniae* infections of mice, (ii) determine the effect of immunological paralysis on cellular mechanisms, and (iii) elucidate the mechanism of immunological paralysis by means of passive cell transfer techniques. As a working hypothesis, it was proposed that cellular immunity operated in this infection and would become more demonstrable when compared to the activity of macrophages from "paralyzed" animals, since this immunological state leads to less than normal resistance (6). Furthermore, this proposal encompassed the supposition that "paralysis" blocked antibody induction due to failure of phagocytes to carry out their suggested inductive role (7, 18) or antigen-processing function (13, 20).

Passive phagocyte transfer experiments showed that increased protection to control and "paralyzed" recipients was derived from "paralyzed" peritoneal cells. One can presently only speculate on effects of these macrophages. It may be that ingested polysaccharide antigen was transferred to control recipients with these cells, providing sufficient antigenic stimulus to invoke an immune response. This would not, however, readily explain why "paralyzed" cells subsequently provided protection to "paralyzed" recipients, since the latter are incapable of forming antibody when exposed again to antigen alone (4, 6). Perhaps in this case, and also in control recipients, the "paralyzed" cells were removed from a donor specific inhibiting environment, allowing production of a "primer" (8) substance in the recipients necessary for the induction of immunity. It may be also assumed that such an inhibitor would be removed during the washing of cells in the transfer procedure. This would obviously require recipients not to form the donor-specific inhibitor.

Another interpretation would assume that antigen concentration within the transferred cells plays a key role. That is, excessive antigen con-

centrations prevailing in "paralyzed" mice after antigen injection are taken up by the phagocytic cells. This may lead to a loss of "priming" function by these cells such that they cannot subsequently enter into the antibody-inducing sequence. Removal of cells from the "paralyzed" host and the washing process could lead to decreased intracellular concentrations through antigen egestion. Subsequent transfer to recipients might then allow induction of protection.

It is noteworthy that "immune" peritoneal macrophages did not provide enhanced resistance to *K. pneumoniae* challenge in control and "paralyzed" recipients (i.e., lack of cellular immunity) by the technique employed. These findings differ from those of Miya, Marcus, and Perkins (14), but different methods were employed. Indeed, preliminary studies of comparative measurements of macrophage activity by *in vitro* methods in our hands indicate positive differences between peritoneal cells of control and immune animals.

"Immune" peritoneal cells were incapable of transferring resistance factors, whereas those from "paralyzed" animals could. If it is assumed that antibody priming activity is involved, it is possible that "immune" cells completed this function prior to transfer. Therefore, recipient animals would derive no benefit from such cells, at least in an antibody-inducing role.

These data also demonstrate that antibody-forming cells are not eliminated in immunologically paralyzed animals, but rather appear to be suppressed. They also indicate that not all protective antibody is bound to antigen *in vivo*, but that there is a central failure of antibody production. Otherwise, antigen would have bound antibody produced by the stimulus of transferred "paralyzed" peritoneal cells, and recipients would have remained susceptible to subsequent challenge.

Data from transfer of spleen cells indicate that such cells from "paralyzed" animals failed to induce protection to recipient control and "paralyzed" mice. This finding is consistent with those of the past (4, 16, 19) that such cells are not actively participating in acquired resistance during "paralysis." Conversely, the general incapacity of spleen cells from immune donor animals to provide increased resistance to control and "paralyzed" recipients offers lack of correlation with earlier literature in studies of *D. pneumoniae* (4, 16). Further investigation will be required to define this difference, although, in the present case, difference in properties of antigens employed may be a factor.

The findings that immunologically paralyzed animals were more susceptible than control

animals was consistent in all of the experiments performed. Although this was reported to be the case in "paralysis" produced by homologous pneumococcal antigen by Felton et al. (6), it had not been shown by others with either *K. pneumoniae* antigens (2) or pneumococcal antigens (17).

Evidence from these studies also indicates that immune paralysis induced by our *K. pneumoniae* antigen persists a finite period of approximately 3 months. Similar effects were reported with "paralysis" induced by *D. pneumoniae* capsular polysaccharide in some instances (1, 15), although other investigators (3, 6) found the condition to exist up to 18 months.

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