Cellular Antigens

Edited by

ALOIS NOWOTNY

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Lectures and Summaries of the
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Edited by

Alois Nowotny

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Preface

Scientists may feel that there are too many meetings these days, and we tend to agree on this until it comes to our own field of interest. In our own areas we would like to hear about other people's achievements and learn whatever may be helpful in the search for answers to our own pet questions. Exchange of ideas, discussions, and critical evaluations are almost as essential to progress as the actual laboratory work. These were the major motives which initiated our own efforts, with the support of Dr. Earle H. Spaulding, the Chairman of the Department of Microbiology and Immunology at Temple University School of Medicine, to bring together for the first time specialists from various disciplines who are attempting to achieve the same thing, *i.e.*, the clarification of the chemical and immunological nature of different cellular antigens.

Instead of publishing the proceedings of the conference, it was decided that we would attempt to review achievements in the different subjects as well as report the latest developments from our own laboratories. Thus we hope to give scientists involved in this explosively growing field not only an up-to-date report, but also a useful source of relevant references.

Despite these efforts and aspirations, we realize that this book is not a complete survey of all known cellular antigens. We tried to present major representations of the most important cell types. By carefully selecting the most prominent specialists on the various topics, we tried to present as many aspects of the subjects as the limitations of time would allow. While immunochemistry dominated the program, many other areas were also discussed from an immunological point of view. If our present plans materialize, we will reconvene in a few years, when we will not only review the newest achievements, but also emphasize those topics of aspects that could not be discussed at our first meeting.

Finally, I would like to express our gratitude to the Ortho Research Foundation and especially to its Vice President, Dr. William Pollack, whose generous support was the sole financial aid which made this conference possible. We also wish to thank all the participants, chairmen, lecturers, and discussants for their valuable contributions, as well as all those who helped us in organizing this meeting.

Last but not least, I wish to acknowledge the superb help of Mrs. June Whitcombe, editorial assistant of our department, and of Mrs. Alice Stone for their enthusiastic and outstanding cooperation.

Philadelphia
March, 1972

ALOIS NOWOTNY

Conference Chairman,
Professor of Immunology,
Temple University School of Medicine

Introduction

The importance of cellular antigens in the homeostasis of the organism as well as in the pathogenesis and natural history of disease is well documented. Currently, there are promising leads suggesting that in addition to their usefulness in diagnosis, they may be applied to the treatment of malignant neoplasms and chronic degenerative diseases. Exploitation of cellular antigens for these purposes requires full understanding of their chemical nature and precise biological function.

The lectures and reviews of the first of a series of meetings on cellular antigens are reported in this volume. The fundamental characteristics and behavior of cellular antigens are presented in breadth and depth. Antigens of bacteria, formed blood elements, normal tissues, and malignant neoplasms, though discussed in separate sessions, are recognized as a continuum. In both the formal presentations and the panel discussions, linkages between model systems, both *in vivo* and *in vitro,* and potential clinical significance are noted. Immunology as a clinical science has, in the past, enjoyed its most spectacular success in the field of infectious diseases. More recently, progress in immunobiology and more specifically immunochemistry has been crucial in facilitating tissue and organ transplantation.

Fundamental knowledge so ably reported by these proceedings is gradually transforming immunochemistry into a rational and useful weapon in the study of the most rapidly growing causes of morbidity and mortality.

Explanations for variations in the occurrence and natural history of cancer and chronic degenerative diseases invoking immunological and host differences are moving from a "mystique" to a science. Accelerated progress from the former to the latter demands the development of models and techniques and elucidation of mechanisms which will hasten the day when the critical components of the immunological equation can be applied to the understanding and control of disease.

The participants in this conference are addressing themselves both as architects and as engineers to the creation of a solid foundation in this most exciting area on the leading edge of biomedical science.

Philadelphia
January, 1972

PAUL KOTIN, M.D.

Vice President of the Health Sciences Center,
Dean of the School of Medicine,
Temple University

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Part 1

Gram-Negative Bacterial Antigens

IMMUNE RESPONSE OF MICE TO SUBCELLULAR VACCINES OF *SALMONELLA TYPHIMURIUM**

L. J. BERRY and M. R. VENNEMAN

Department of Microbiology, The University of Texas at Austin, Texas

One of the major unanswered questions of immunology is why a living bacterial vaccine affords better protection than any of the currently available killed vaccines. It has been suggested that this is due either to a labile antigen (or antigens) no longer present in killed preparations or that it results from a more continuous and hence effective antigenic stimulation, probably through a limited proliferation of the pathogen. A distinction between the two possibilities would be possible only if the labile antigen could be identified.

My entry (L. J. B.) into this area of research was predicated on years of work with experimental mouse typhoid and was inspired by the important observations of YOUMANS and YOUMANS (1964, 1965, 1966) to the effect that a ribosomal vaccine derived from *Mycobacterium tuberculosis* provided impressive protection against tuberculous challenge in mice. You will hear an account of their studies, but it is appropriate for me at this time to express my indebtedness to them. Working with me at Bryn Mawr College at the time of our initial experiments were Ruth Levy, Toby Eisenstein, and Stuart Winston.

At Ohio State University, Dr. Nancy Bigley and Martin Venneman were similarly motivated by the Youmans' work to initiate studies with *Salmonella typhimurium*. Their findings (VENNEMAN and BIGLEY, 1969; VENNEMAN *et al.*, 1970) and those of ours (EISENSTEIN *et al.*, 1968; WINSTON and BERRY, 1970a, b; EISENSTEIN and BERRY, unpublished observations) were in essential agreement. Dr. Venneman first joined me at Bryn Mawr College as a postdoctoral fellow and then made the move with

* The work described in this report was supported in part by grants from the National Institute of Allergy and Infectious Diseases, the National Science Foundation, and Smith, Kline, and French Laboratories.

me to Texas. In my remarks, I will try to highlight our results to date (VENNEMAN and BERRY, 1971a, b, c).

Table 1 summarizes our most important initial results. CF-1 mice or a similar Swiss Webster strain were used throughout the studies. Vaccines were given subcutaneously and challenge was via the intraperitoneal route. The use of an adjuvant resulted in a slight increase in level of immunity achieved with the preparations used here but not with the highly purified RNA, as will be demonstrated below.

Table 1.* Protective Effect in Mice of a Single Immunizing Dose of Different Vaccines Followed 15 Days Later by Challenge with 1000 LD_{50}'s of *Salmonella Typhimurium*

Vaccine	Dose	Living/Total after 30 days	Percent survival
RIA	0.1 LD_{50}	37/37	100
Heat-killed *S. typhimurium*	50 μg	13/62	21
Ribosomal fraction	100 μg	36/36	100
	10 μg	39/45	82
RNA† fraction	100 μg	83/90	92
	10 μg	139/150	92
Control		55/280	20

* Modified from VENNEMAN and BIGLEY (1969) and VENNEMAN *et al.* (1970).

† Derived from the ribosomal fraction.

RIA is the designation of a strain of *S. typhimurium* of low virulence. It was obtained originally—as was our highly virulent challenge strain, SR-11—from Dr. Howard Schneider, then at the Rockefeller University. The heat-killed vaccine was prepared by heating the SR-11 strain to boiling point for 30 minutes, or to 63°C for 30 minutes. Both gave similar results.

The ribosomal fraction was obtained by a procedure similar to that used by YOUMANS and YOUMANS (1965). The flow diagram presented in Figure 1 indicates the steps employed.

Cells of *S. typhimurium*, SR-11 (Figure 1), grown overnight, were ruptured with a French pressure cell. Whole cells and debris were sedimented at 22,000 × g for 10 minutes and 43,000 × g for 15 minutes, and the supernatant was sedimented at 105,000 × g for 3 hours. Following treatment of the pellet with 0.5% sodium dodecylsulfate (SDS) for 30 minutes at room temperature, the material obtained after 3 hours centrifugation at 105,000 × g was designated the ribosomal fraction.

The initial RNA fraction was obtained, as indicated by the flow diagram in Figure 2, by extracting the first high-speed pellet (the ribosomal

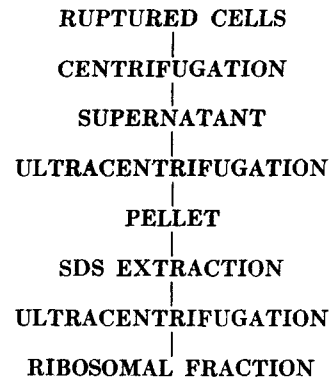

Fig. 1. Flow diagram for preparation of ribosomal fraction.

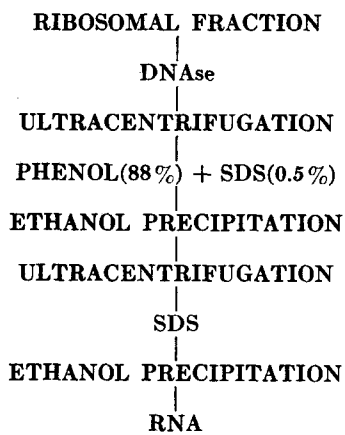

Fig. 2. Flow diagram for extraction of ribosomal RNA.

fraction) for 10 minutes at 65°C with 88% phenol and 0.5% SDS. Extraction with phenol was repeated four times at 25°C. The RNA was precipitated with ethanol, collected by high-speed centrifugation, treated again with SDS to decrease further the protein and endotoxin content of the material, and again precipitated with ethanol.

The challenge dose for the data presented in Table 1 was 1000 LD_{50} of SR-11. High levels of protection were afforded by the living vaccine and by both the ribosomal and RNA preparations each at two dose levels. Even with 1 μg of the ribosomal or the RNA vaccine, highly significant levels of immunity, not shown in Table 1, were evident.

The lack of protection seen with the killed vaccine is explainable in part by the high challenge dose. Even though the survival time of mice

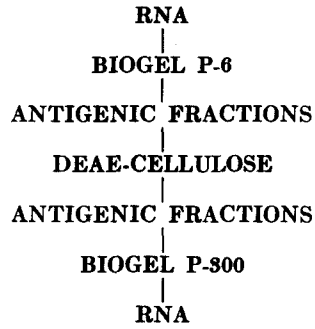

Fig. 3. Flow diagram for purification of ribosomal RNA.

immunized with this vaccine was greater than that of controls, the percentage alive at 30 days was not.

We shall omit description of earlier studies and analyses aimed at identifying the active component in the RNA vaccine and go directly to the latest work, which makes it look as if the active immunogen is, in fact, RNA. Figure 3 is a flow diagram of the procedures used in obtaining a highly purified RNA. The last Biogel excludes substances of 300,000 or more molecular weight.

Table 2 summarizes the results of chemical analyses of the purest RNA preparation so far obtained. It is evident that any material other than RNA (with the possible exception of carbohydrate) would have to

Table 2.* Chemical Analyses of the Purified Ribosomal RNA from *Salmonella Typhimurium*

Material sought	Assay method	Amount of RNA assayed (dry wt)	Amount present
Protein	Analytical Polyacrylamide Disc Electrophoresis	2 mg	<0.5 μg (none detected)
	Lowry	50 mg	
DNA	Diphenylamine	50 mg	<0.5 μg (none detected)
Lipid	Thin-layer chromatography	300 μg	<0.5 μg (none detected)
RNA	Orcinol	50 mg	"100%"
Carbohydrate	Pentose equivalents	50 mg	20–25%

* Modified from Venneman (submitted for publication).

be a superantigen, were it responsible for protection. This becomes apparent from the data in Table 3. The "purified" RNA protects extremely well two weeks after an immunizing dose of 50 ng. Were protein responsible, the most that could be present would be less than 0.5 ng. It is apparent that incorporating the purified RNA in Freund's incomplete adjuvant failed to increase its protective effect. To this extent, our results are different from those of YOUMANS and YOUMANS (1966), who find adjuvants essential for protection.

Table 3.* Comparison of the Protective Effect of a Living Vaccine with Ribosomal RNA Vaccines in Mice

Vaccine	Diluent	Living/Total after 30 days	Percent survival
RIA (living)	Saline	35/40	90
"Crude" RNA (50 μg)	Saline	35/40	90
	Adjuvant†	32/40	80
"Purified" RNA (50 μg)	Saline	38/40	95
	Adjuvant	34/40	85
Controls		4/40	10

* Modified from Venneman (submitted for publication).
† Freund's incomplete adjuvant.

With the above evidence implicating RNA as an active antigen capable of conferring a level of protection equal to that obtained with a living vaccine, attention should now be directed to another aspect of the work.

One of the long-sought goals of immunology has been to distinguish between the relative contribution of cellular and humoral immunity to an animal's resistance to disease. Can there, in fact, be cellular immunity without antibody involvement? One of the major obstacles to obtaining an answer is methodology. How can one clearly separate one types of immunity from the other? Some outstanding investigators have addressed themselves to this problem. ROWLEY *et al.* (1964) and JENKIN and ROWLEY (1963) in Australia, ELBERG (1960) in Berkeley, California, MACKANESS (1964) and his group at the Trudeau Institute, in Saranac Lake, N.Y., and SAITO *et al.* (1962) in Japan serve as an incomplete but illustrative list.

The ideal way to separate cellular immunity from humoral immunity requires a technique for the initiation of one without the other. Assuming that this were possible, what test of immunity should be used to evaluate the relative resistance of the host to challenge? There are, in general, two approaches. One relies on survival and the other on the ability of immunized-versus-control animals to suppress the *in vivo* growth of the

pathogen. We have done both, but the latter procedure saves time—at least three weeks per experiment.

Our design was to infect mice two weeks or 15 days postvaccination with multiple LD_{50}'s of the SR-11 (virulent) strain of *S. typhimurium.* Viable pathogen counts from whole mouse homogenates were then followed with time postinfection, a technique described some years ago by BERRY *et al.* (1956). Since controls began to die after 5 days, this time was chosen as the standard interval when all counts were made. The data presented in Table 4 are typical when mice were challenged 15 days post-

Table 4.* Viable Counts of *Salmonella Typhimurium* in Whole Mouse Homogenates 5 Days Postchallenge in Control and Vaccinated Mice

Vaccine	Log of mean number of bacteria/g mouse
None	5.7
Heat-killed *S. typhimurium*	3.0
Ribosomal RNA	3.1

* Modified from VENNEMAN and BERRY (1971a,b).

immunization. They had been injected with 20 μg of either a killed vaccine or 20 μg of the "purified" ribosomal RNA. The challenge dose was 40 LD_{50}'s. Each value is the log of the mean of 10 to 15 separate determinations. The suppression of growth was equal in vaccinated mice, regardless of the immunogen employed, and in each group there were about 1/500 as many salmonellae as in controls. The ability of mice given the killed vaccine to control the pathogen count is probably related to the use of the small challenge dose of 40 LD_{50}'s. In Table 1, mice that were immunized with the killed vaccine failed to survive better than controls, but the challenge dose in that series of experiments was 1000 LD_{50}'s. The level of protection may vary, therefore, depending on the immunogen administered, but only with large infectious doses may the differences become evident.

The next step in our studies was to evaluate the ability of serum or of peritoneal cells from mice previously immunized with one of the vaccines to passively protect normal recipient animals against challenge as determined by pathogen count. In Figure 4, a diagram of the experimental design is presented. Peritoneal macrophages were removed without any attempt to separate them into cell types. The mice, it should be recalled,

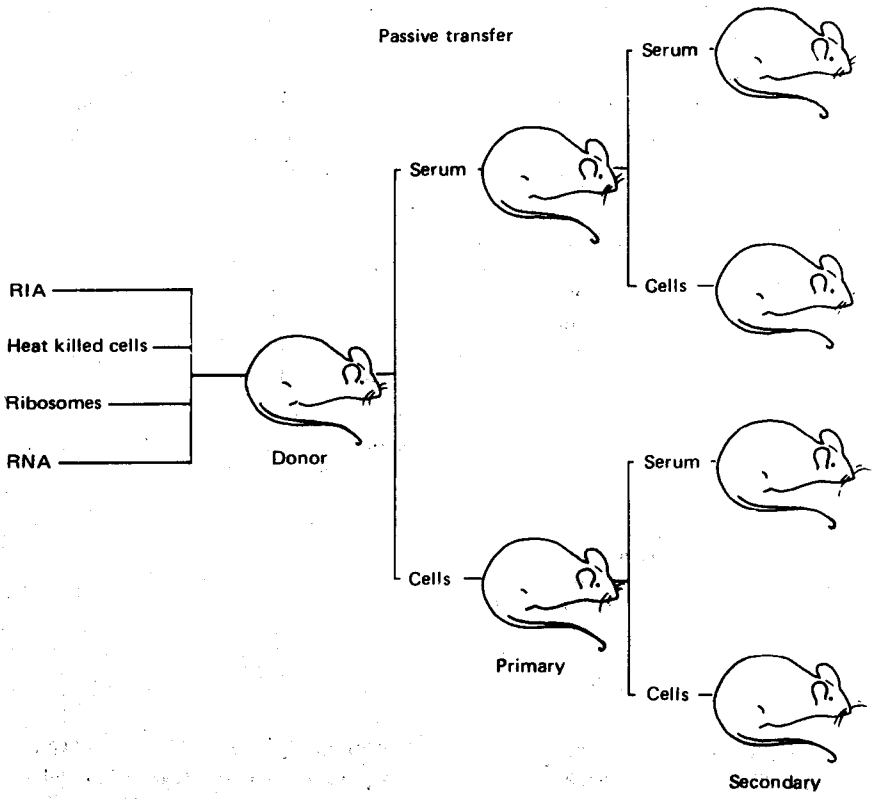

Fig. 4. Diagram of procedure for primary and secondary passive transfer of immunity with peritoneal cells or serum.

are outbred. Cells were injected intraperitoneally, and the recipients were challenged 15 days later via the same route. Pathogen counts were made 5 days after that.

An 0.1 ml amount of serum was injected intraperitoneally, and the mice were challenged 7 days later via the same route. Pathogen counts were made 5 days after challenge.

For secondary transfer, cells were removed from primary recipients 15 days posttransfer, and the new recipients were challenged 15 days later. For serum, transfer from primary recipients to secondary recipient was at 7 days and challenge at 4 days. Table 5 shows the log of average number of pathogens in mice challenged with 45 LD_{50}'s after the mice in all groups had received 10^5 peritoneal cells 15 days before infection. The controls received peritoneal cells from normal mice. The donor mice were vaccinated 15 days earlier with 20 μg of one or the other antigenic materials. The pathogen counts were made 5 days postinfection. Each value is the