Current Topics in Microbiology and Immunology

85

Edited by

W. Arber, Basle · S. Falkow, Seattle · W. Henle, Philadelphia
P.H. Hofschneider, Martinsried · J.H. Humphrey, London · J. Klein,
Tübingen · P. Koldovský, Düsseldorf · H. Koprowski, Philadelphia
O. Maaløe, Copenhagen · F. Melchers, Basle · R. Rott, Gießen
H.G. Schweiger, Ladenburg/Heidelberg · L. Syruček, Prague
P.K. Vogt, Los Angeles

With 41 Figures

Springer-Verlag
Berlin Heidelberg New York 1979

ISBN-13: 978-3-642-67324-5 e-ISBN-13: 978-3-642-67322-1
DOI:10.1007/978-3-642-67322-1

This work is subject to copyright. All rights are reserved, whether the whole or part of the material is concerned, specifically those of translation, reprinting, re-use of illustrations, broadcasting, reproduction by photocopying machine or similar means, and storage in data banks.
Under §54 of the German Copyright Law where copies are made for other private use, a fee is payable to the publisher, the amount of the fee to be determined by agreement with the publisher.
© by Springer-Verlag Berlin Heidelberg 1979.
Softcover reprint of the hardcover 1st edition 1979
Library of Congress Catalog Card Number 15-12910.

The use of registered names, trademarks, etc. in this publication, does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
Typesetting, printing and binding: Universitätsdruckerei H. Stürtz AG, Würzburg
2121/3321-543210

Table of Contents

Immunology of the Nicotinic Acetylcholine Receptor

SARA FUCHS [1]

I. Introduction

The nicotinic acetylcholine receptor (AChR) has been extensively studied both from the structural and biologic points of view. It is the only receptor for a neurotransmitter that has been purified and can be prepared in substantial amounts. Characterization of this component has been greatly advanced by the availability of AChR in large quantities in electric organs of electric fish (*Nachmansohn*, 1959) and by the use of α-neurotoxins from elapid snake venoms which bind specifically and with a high affinity to the nicotinic acetylcholine receptor (*Lee*, 1972).

The biochemical and pharmacologic properties of AChR as well as the procedures for its purification have been summarized in a number of review articles (*Changeux*, 1975; *Heilbronn*, 1975; *Raftery* et al., 1976; *Karlin* et al., 1976; *Briley* and *Changeux*, 1977) and will not be discussed in this review. Special interest in the immunologic properties of AChR arises from the involvement of an autoimmune response to this receptor in the human neuromuscular disease, myasthenia gravis. In addition, antibodies to AChR are a useful tool for studying molecular and biologic aspects of AChR. It is to these aspects of AChR that this review is devoted.

[1] Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel

II. Immunochemical Analysis of AChR

Immunization of rabbits, mice, rats, guinea pigs, and monkeys with AChR purified from electric fish results in the formation of antibodies. Earlier works utilized AChR from *Electrophorus electricus* for immunization (*Patrick* and *Lindstrom*, 1973; *Sugiyama* et al., 1973; *Aharonov* et al., 1975b) and more recently AChR from *Torpedo* has been used extensively for immunization and immunochemical studies, as well as for the induction of experimental myasthenia. Immunochemical analysis of AChR was carried out mostly on rabbit antisera. Different methods were applied for characterizing anti-AChR antibodies. These included direct quantitative precipitin reactions (*Aharonov* et al., 1975b; *Bartfeld* and *Fuchs*, 1977; *Green* et al., 1975; *Sugiyama* et al., 1973; *Penn* et al., 1976), immunodiffusion and immunoelectrophoresis (*Aharonov* et al., 1975b, 1977), microcomplement fixation (*Aharonov* et al., 1975b), passive microhemagglutination (*Fuchs* et al., 1976), and radioimmunoassays in which the labeled antigen was ^{125}I-AChR (*Aharonov* et al., 1977), ^{125}I-toxin-AChR, ^{3}H-acetyl-AChR (*Patrick* et al., 1973), or ^{3}H-MBTA-AChR (*Karlin* et al., 1976). In all radioimmunoassay procedures the antigen-antibody complexes formed were precipitated with goat anti-immunoglubulin sera.

AChR ist an immunopotent immunogen and gives rise to precipitable antibodies that display a single precipitin line with purified AChR in immunodiffusion (*Aharonov* et al., 1977). There is no cross reactivity between AChR and the closely related membrane protein, acetylcholinesterase (*Sugiyama* et al., 1973; *Aharonov* et al., 1975b). On the other hand, AChR is highly conserved during evolution and there is an immunologic cross reactivity between AChR of different species (*Fuchs* et al., 1976; *Tarrab-Hazdai* et al., 1977a; *Aharonov* et al., 1977; *Prives* et al., 1978). The degree of cross reactivity obtained depends on both the phylogenetic distance between receptors tested as well as on the sensitivity of the assay used. Antibodies against eel receptor cross react with *Torpedo* and with chicken receptor (*Sugiyama* et al., 1973). Cross reactivity between *Torpedo* and eel receptor was reported also by *Penn* et al. (1976) and by *Karlin* et al. (1976). Immunologic cross reactivity was also observed between fish and mammalian skeletal muscle AChR (*Aharonov* et al., 1977; *Fuchs* et al., 1976; *Bevan* et al., 1976; *Green* et al., 1975). Such cross reactivity is a crucial requirement for the induction of an immunologic block by fish AChR in experimental autoimmune myasthenia gravis. Antibodies elicited against fish AChR were shown to cross react with nicotinic receptor in the central nervous system (*Patrick* and *Stallcup*, 1977; *Tarrab-Hazdai, Edery, Fuchs,* unpublished data). Cross reactivity between muscle and brain AChR has also been observed in other studies (*Fulpius* et al., 1977; *Lefvert* and *Pirskanen*, 1977).

Anti-AChR antibodies bind to ^{125}I-toxin labeled AChR, indicating that antibodies directed against determinants other than the acetylcholine-binding site are present (*Aharonov* et al., 1977). Moreover, the relationship between the antigenic and toxin-binding sites of AChR was studied by experiments measuring the inhibition by cholinergic ligands and α-toxins of the binding of ^{125}I-AChR to anti-*Torpedo* AChR antibodies. No inhibition of the binding

was observed by any of the following materials: carbamylcholine, *d*-tubocurarine, decamethonium, hexamethonium, atropine, *Naja naja siamensis* α-toxin, or α-bungarotoxin, suggesting that no antibodies were produced against the toxin-binding or acetylcholine-binding site of the receptor. Thus the antigenic sites of the receptor molecule are different from the toxin-binding and physiologic sites (*Aharonov* et al., 1977).

The finding that anti-AChR sera do not contain antibodies specific to the toxin-binding or the acetylcholine-binding site suggests that this site (or sites) are not immunodominant determinants. Since the acetylcholine-binding site has to be on the surface of the receptor molecule so as to perform its physiologic function of binding acetylcholine and the toxin-binding site is either identical with or adjacent to it, they would both be expected to be available also to the immune system. The acetylcholine-binding site is probably a highly conserved region of the AChR in evolutionary terms and it is not, therefore, surprising that the immunized animal recognizes it as "self" and does not produce antibodies against it.

Although anti-AChR antibodies are not directed against the toxin-binding site (see also *Patrick* et al., 1973) they can nevertheless block both toxin-binding (*Aharonov* et al., 1977) and the physiologic activity of the receptor (*Patrick* et al., 1973; *Sugiyama* et al., 1973; *Green* et al., 1975). This blockage may be caused by antibodies to antigenic determinants located close to the physiologically active site, e.g., on an adjacent part of the same polypeptide chain, or brought into proximity either by folding or by juxtaposition of sites on different subunits. Alternatively the blockage may be of an allosteric nature by combination of antibodies at a site distant from the acetylcholine-binding site. In addition, antibodies may block the physiologic activity of the receptor by interfering with the regulation and/or function of the ionophore moiety of the receptor molecule. It is possible that only antibodies that block the toxin-binding or the physiologic activity of the receptor are those that play a role in the myasthenic action of AChR (*Bartfeld* and *Fuchs*, 1977, 1978; *Zurn* and *Fulpius*, 1977).

The immunochemical properties of the detergent-purified AChR molecule appear to be indistinguishable from those of the receptor in the crude extract (*Aharonov* et al., 1977) as well as from those of purified water-soluble preparation of AChR not exposed to detergents (*Aharonov* et al., 1975b), or the receptor in AChR-rich membrane fraction (*Tarrab-Hazdai* and *Fuchs*, unpublished data). Immunization of rabbits with AChR-rich membrane fraction (*Cohen* et al., 1972) in which probably only part of the AChR molecule is immunogenically exposed, gives rise to antibodies of similar specificity to those obtained by immunization with detergent-purified AChR and also leads to the development of experimental autoimmune myasthenia gravis (*Tarrab-Hazdai* and *Fuchs*, unpublished data).

Specific anti-AChR antibodies were purified from anti-AChR sera by immunoadsorption on AChR-Sepharose columns followed by elution from the column with 0.1 *M* NH_4OH and dialysis against phosphate-buffered saline (*Martinez* et al., 1977). The purified antibodies could bind ^{125}I-AChR and gave a single band of precipitation in immunodiffusion when tested against AChR or goat anti-rabbit IgG serum. Antibodies of some specificity did not seem to be

adsorbed onto AChR-Sepharose columns, possibly due to a destruction of some antigenic determinants during the covalent binding of AChR to the Sepharose. In order to overcome this problem an indirect method was employed for the preparation of AChR immunoadsorbent which involved a strong noncovalent binding of AChR (*Schwartz* et al., 1978a). α-Bungarotoxin was first attached to Sepharose and the resulting toxin-Sepharose was complexed with AChR. Anti-AChR serum was applied to the AChR-toxin-Sepharose column and the adsorbed antibodies were eluted with 0.1 *M* NH_4OH and dialyzed against phosphate-buffered saline. All the anti-AChR antibody activity was adsorbed to the column and recovered in the eluted antibody fraction. Furthermore, the purified antibodies were capable of inhibiting the binding of radiolabeled toxin to AChR, indicating that at least part of the antigenic determinants of AChR which are associated with toxin-binding sites were free in the immunoadsorbent column (*Schwartz* et al., 1978a). This latter procedure for isolation of purified anti-AChR antibodies is now routinely employed in our laboratory with satisfactory results.

Further immunochemical analysis of AChR and a correlation with structural features of the molecule were achieved by studying chemically modified AChR or other derivatives of this molecule. Chemical modification of free amino groups of the receptor molecule was achieved by reacting purified AChR with N-carboxy-DL-alanine anhydride to form poly-DL-alanyl AChR (PA-AChR) (*Schmidt-Sole* et al., 1977). The PA-AChR preparation had an average of five additional alanine residues per lysine residue and bound toxin to the same extent as the unmodified receptor. Although this chemical modification did not affect the physiologic activity of the receptor, it abolished the myasthenic activity. Polyalanylation resulted in a marked change in the immunogenicity but not in the antigenic specificity of AChR. Rabbits immunized repeatedly with PA-AChR did not show any clinical signs of experimental autoimmune myasthenia gravis. Antibodies from rabbits immunized with PA-AChR were directed mainly to the polyalanine determinants and bound AChR only to a very limited extent. On the other hand, the reactivity of PA-AChR towards anti-AChR antibodies was as efficient as that of AChR (Fig. 1) (*Schmidt-Sole* et al.,

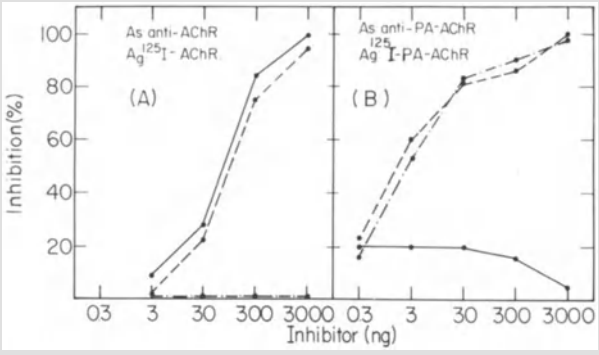

Fig. 1. Specificity of anti-AChR and anti-PA-AChR antibodies. Inhibition of the binding of: (A) ^{125}I-AChR to anti-AChR serum and (B) ^{125}I-PA-AChR to anti-PA-AChR serum by AChR (——), PA-AChR (– – –), and PA-BSA (-·-·-)

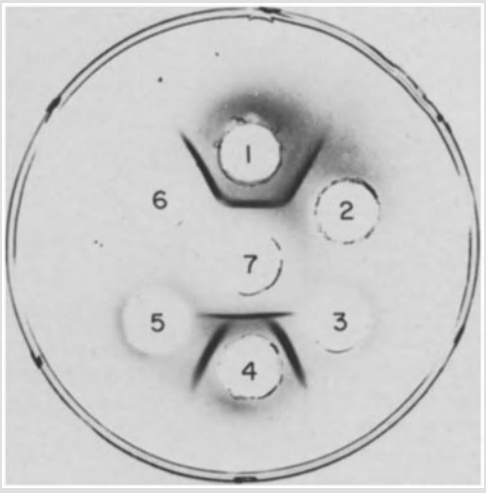

Fig. 2. Immunodiffusion of anti-RCM-AChR serum (*well 1*) and anti-AChR serum (*well 4*) with RCM-AChR (*wells 2, 3 and 5*) and AChR (*well 6 and 7*). (*Bartfeld* and *Fuchs*, 1977)

1977). Such immunologic specificity may render PA-AChR an effective agent for immunosuppresion of EAMG by antigenic competition or, alternatively, by neutralizing the immune response to AChR.

Irreversibly denatured AChR derivative was obtained by complete reduction and carboxymethylation of AChR in 6*M* guanidine hydrochloride (*Bartfeld* and *Fuchs*, 1977). The reduced carboxymethylated receptor (RCM-AChR) lost the pharmacologic activity of the intact AChR and did not show any detectable binding of ^{125}I-α-bungarotoxin. RCM-AChR lost also the myasthenic activity of AChR, for rabbits repeatedly immunized with RCM-AChR did not develop any clinical signs of EAMG. Nevertheless, anti-RCM-AChR sera cross react with AChR (*Bartfeld* and *Fuchs*, 1978; *Fuchs* et al., 1978).

Comparison of the specificity of anti-AChR with that of anti-RCM-AChR has indicated some qualitative differences between the two antisera. By both immunodiffusion and radioimmunoassay measurements an identity was observed between the reaction of anti-RCM-AChR with RCM-AChR and AChR. However, anti-AChR serum showed only a partial cross reactivity with RCM-AChR (Figs. 2 and 3). RCM-AChR and AChR inhibited the binding of ^{125}I-AChR to anti-RCM-AChR to the same extent, whereas RCM-AChR was a weaker inhibitor than AChR of the binding of ^{125}I-AChR to anti-AChR and bound to only part of these antibodies. In addition to these differences it was also observed that whereas anti-AChR antibodies block the in vitro binding of ^{125}I-α-Bgt to AChR effectively, anti-RCM-AChR antibodies blocked this binding only to a very limited extent (*Bartfeld* and *Fuchs*, 1977; *Fuchs* et al., 1978).

Analysis of both the intact and denatured receptor suggested that some antigenic determinants in the AChR molecule were abolished by the denaturation procedure. However, no additional determinants that were not expressed in the intact molecule became immunopotent after reduction and carboxymethyla-

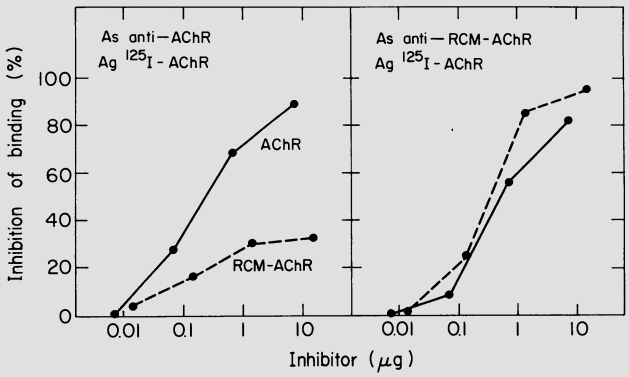

Fig. 3. Specificity of anti-AChR and anti-RCM-AChR antibodies. Inhibition of the binding of ^{125}I-AChR to anti-AChR serum (*left*) and anti-RCM-AChR serum (*right*) by AChR (——) and RCM-AChR (– – –) (*Bartfeld* and *Fuchs*, 1978)

tion. The altered antigenic specificity of antibodies to RCM-AChR along with their altered effect in blocking toxin binding to AChR led us to propose that the denaturation of AChR destroyed some antigenic determinant(s) that is (are) important for the induction of EAMG and may be located close to the toxin-binding site. The cross reactivity between AChR and RCM-AChR and the nonpathogenicity of the latter appear to be crucial in governing the immunosuppressive and therapeutic effects of RCM-AChR on EAMG (see below, Sect. 4; *Fuchs* et al., 1978; *Bartfeld* and *Fuchs*, 1978).

A similar pattern of cross reactivity between AChR and denatured AChR was also obtained by employing SDS-denatured AChR preparation (*Karlin* et al., 1976). Detailed analysis of antigenic determinants in the AChR molecule can be achieved by studying the immunogenicity and antigenic specificity of the individual polypeptide chains of AChR. Four polypeptide chains were isolated and purified from *Torpedo californica* AChR by preparative acrylamide gel electrophoresis (*Claudio* and *Raftery*, 1977; *Lindstrom* et al., 1978). Antibodies against each isolated subunit cross react with AChR, indicating that none of the subunits is entirely buried in the intact AChR molecule. The four polypeptide chains were reported to be immunogenically distinct from one another (*Claudio* and *Raftery*, 1977; *Lindstrom* et al., 1978).

It should be pointed out that a higher cross reactivity seems to exist between denatured AChR molecules from different sources than that observed between native AChR molecules. The higher cross reactivity on the denatured level of proteins was also reported for other immunogens (*Arnon* and *Maron*, 1971) and may be particularly relevant for experiments of specific immunosuppression of AChR-induced myasthenia (see below, Sect. 4; *Bartfeld* and *Fuchs*, 1978).

III. Antibodies to AChR as Tools

Antibodies against AChR provide a useful tool for investigating several structural and biologic aspects of AChR. This approach has great potential due

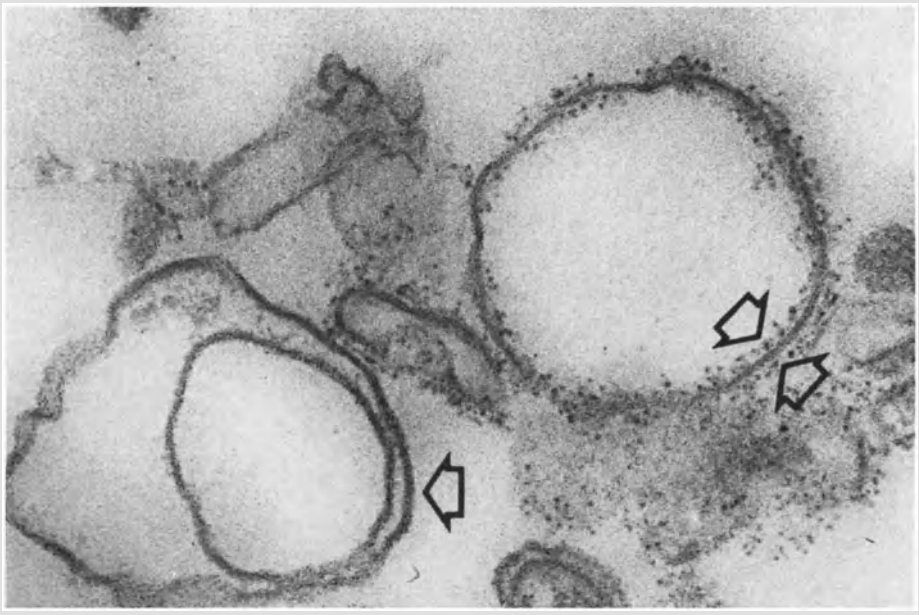

Fig. 4. Immunoferritin labeling of AChR in membrane preparation from *Torpedo californica*. Ferritin molecules stain both sides of the membrane profile (*opposing arrows*). In contrast, profiles devoid of staining (single *arrow*) probably represent receptor-free vesicles. The detailed procedure for the immunoferritin staining is described elsewhere (*Tarrab-Hazdai* et al., 1978a). $\times 90{,}000$

to both the interspecies cross reactivity between nicotinic receptors and to the availability of antibodies against distinct regions of the AChR molecule. A few examples on the application of anti-AChR antibodies as tools for studying localization, distribution, and characterization of AChR in the excitable membrane will be described.

The immunoferritin technique using anti-AChR antibodies proved to be an appropriate tool for studying the mode of insertion of AChR in the excitable membrane and for testing whether or not antigenic sites of AChR are exposed on both sides of the membranes. Nicotinic acetylcholine receptor was localized in receptor-rich membrane preparation from the electric organ of *Torpedo californica* by immunoferritin staining, using anti-AChR antibodies against highly purified AChR (*Tarrab-Hazdai* et al., 1978a). The membrane preparation was incubated with $(Fab')_2$ fragments derived from rabbit anti-AChR antibodies and subsequently with ferritin-conjugated goat anti-rabbit immunoglobulin. With this technique more than 50% of the membrane vesicles were found to be labeled with ferritin, while the rest remained unlabeled. Ferritin labeling on both sides of the membrane was evident in open membrane vesicles and occasionally on closed membrane profiles (Fig. 4); the latter may represent unsealed vesicles in which the damage to the vesicle was outside the plane of sectioning. The labeling in closed vesicles was confined to the outer surface of the membrane due to the inability of the antibodies to penetrate the mem-

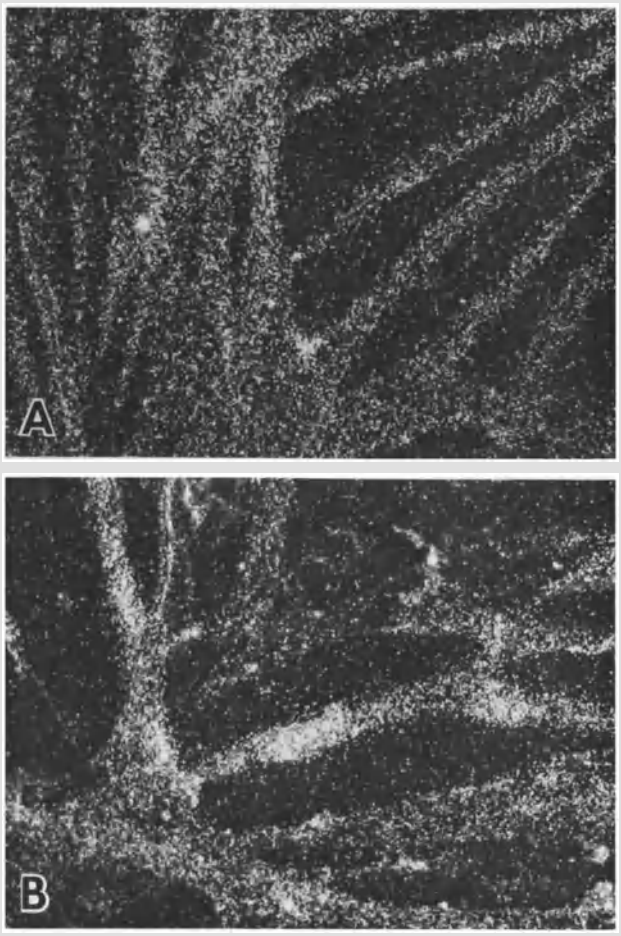

Fig. 5. Aggregation of AChR in cultured muscle cells by anti-AChR antibodies. 96-h-old cultures of chick embryo muscle cells were labeled for 60 min with ^{125}I-α-bungarotoxin and subsequently incubated for additional 4 h at 37° C with (A) 10% of normal rabbit serum and (B) 10% of rabbit anti-*Torpedo* AChR. Note the uniform labeling of the myotubes with silver grains in *a* and the appearance of several large clusters of grains (~50 μm in size) on the surface of mytobues in *b*. The processing of the cultures for autoradiography was specified elsewhere (*Prives* et al., 1976). Dark field optics. ×180

brane. The data suggest that antigenic sites of the receptor molecule are exposed on both sides of the excitable membrane and that acetylcholine receptor is a transmembrane protein (*Tarrab-Hazdai* et al., 1978a). The cross reactivity between antibodies to *Torpedo* AChR and AChR of other species permits the use of such antibodies as a tool for fine localization of AChR in other species as well.

Antibodies against *Torpedo* AChR were utilized for determining which factors affect the stability and distribution of acetylcholine receptors on surface membranes of chick embryo muscle cells in culture (*Prives* et al., 1978). Anti-

Torpedo AChR antibodies were shown to bind to AChR in differentiating skeletal muscle cells. Increased rate in the degradation of AChR was found to result from incubation of muscle cell cultures with anti-AChR immunoglobulins or with the divalent $(Fab')_2$ fragments derived from it (*Prives* et al., 1978). Such an acceleration in the degradation of AChR was also reported as a result of application of immunoglobulins of myasthenic patients on cultures of rat skeletal muscle cells (*Kao* and *Drachman*, 1977a). The accelerated degradation of AChR by anti-AChR antibodies was shown to be associated with formation of large AChR aggregates on the muscle cell surface (Fig. 5). Monovalent Fab fragments of the specific immunoglobulins did not affect the surface distribution of AChR or its rate of degradation (*Prives* et al., 1978). Rabbit anti-fish AChR antibodies were also used for identifying AChR and for following its biosynthesis during differentiation of cultured muscle cells (*Merlie* et al., 1975; *Prives* and *Hoffman*, 1978). The antibodies were utilized to precipitate specifically ^{35}S-methionine-labeled AChR. Anti-fish AChR antibodies also precipitated ^{35}S-methionine-labeled extrajunctional AChR of denervated rat diaphragm muscle and could not distinguish extrajunctional from junctional AChR (*Brockes* and *Hall*, 1975).

The identification of nicotinic AChR or cross-reacting antigens in organs other than skeletal muscle, e.g., brain and thymus, was assessed by using anti-electric fish AChR. Anti-electric eel AChR antibodies were found to inhibit sodium flux in rat sympathetic neuron cell line. Detergent extracts of these cells inhibited the precipitation of muscle AChR with antiserum against eel AChR (*Patrick* and *Stallcup*, 1977). Thus ganglionic nicotinic receptors and muscle nicotinic receptors appear to share antigenic determinants. The immunologic cross reactivity between muscle and brain AChR may have some significance for the possible involvement of AChR of the central nervous system in myasthenia gravis and experimental autoimmune myasthenia gravis (*Fulpius* et al., 1977; *Lefvert* and *Pirskanen*, 1977). We have recently shown that the interaction of anti-skeletal muscle AChR antibodies with brain AChR leads to neurologic disorders not necessarily associated with myasthenia (*Tarrab-Hazdai, Edery, Fuchs*, unpublished data).

Cross reactivity between AChR and the thymus was demonstrated by using anti-AChR antibodies (*Aharonov* et al., 1975c). Immunologic cross reactivity, both cellular and humoral, was observed between AChR from *Electrophorus electricus* and calf thymus fractions (*Aharonov* et al., 1975c). The cross reaction between AChR and thymic components may explain the association between end plate and thymus disorders in myasthenia. This will be discussed in some more detail in Sect. 4.

IV. Experimental Autoimmune Myasthenia Gravis

Since the early 60s myasthenia gravis (MG) has been proposed to be an autoimmune disorder involving an immunologic response to a protein in the neuromuscular junction (*Nastuk* et al., 1960; *Simpson*, 1960). However, in spite of increasing evidence in support of an autoimmune basis for the disease its exact origin

was unknown. The reports in 1973 about the decrease in postsynaptic acetylcholine receptors in MG (*Fambrough* et al., 1973) and about the induction in rabbits of symptoms resembling those of MG following injections with purified AChR from electric eel (*Patrick* and *Lindstrom*, 1973) shed new light on this issue. Considerable evidence now exists that MG is indeed an autoimmune disease in which AChR is a major autoantigen and that the experimental model disease induced by AChR and designated experimental autoimmune myasthenia gravis (EAMG) is an appropriate model for the human disease. The similarity between the human and experimental diseases and the involvement of AChR in both of them permits detailed studies on the pathogenesis and therapy of MG. In the following sections I will review some studies along these directions. Besides the immediate significance of such studies for MG, they are also valuable for the general understanding of autoimmune phenomena in man.

A. Induction of Experimental Autoimmune Myasthenia Gravis (EAMG)

EAMG has been induced in various animal species such as rabbits, rats, guinea pigs, monkeys, and mice by injection of purified AChR from electric organ tissues of *Electrophorus electricus* or from *Torpedo* (*Patrick* and *Lindstrom*, 1973; *Sugiyama* et al., 1973; *Tarrab-Hazdai* et al., 1975a, b, c; *Heilbronn* et al., 1975; *Green* et al., 1975; *Lennon* et al., 1975; *Fuchs* et al., 1976). The induction of EAMG by purified mammalian AChR (*Granato* et al., 1976) and by syngeneic preparations of AChR (*Lindstrom* et al., 1976a) has also been reported. In all cases immunization with the receptor has been performed in adjuvants for obtaining clinical myasthenic symptoms. The disease in rabbits, monkeys, and occasionally in mice seems to be acute and fatal, whereas the disease in rats, guinea pigs, and mice is mild and transient.

EAMG in rabbits is in most cases induced following a single immunization with purified AChR. In a typical experiment rabbits (weighing 2–2.5 kg) were injected once in the hind foot pads and intradermally at three or four sites with purified AChR (80–100 μg) emulsified with an equal volume of Freund's complete adjuvant (*Aharonov* et al., 1977). After 3–4 weeks the rabbits showed signs of fatigue, hypoactivity, anorexia, and weight loss. A few days later the rabbits displayed extreme, flaccid paralysis of limbs and trunk, severe difficulties in breathing, uncontrolled salivation, and severe dysphagia. The rabbits died within a week after the appearance of clinical signs. These clinical signs were temporarily reversed following intravenous injection of the anticholinesterase, edrophonium hydrochloride (Tensilon, 0.5 mg in 1 ml saline). At the most severe stage of the disease this test was not effective, a finding suggesting that irreversible damage had been caused. Electromyograms from sick animals showed a decremental myasthenic response to repetitive nerve stimulation. Humoral and cellular immune responses against the immunizing fish AChR as well as against rabbit skeletal muscle AChR were observed in all the animals (*Aharonov* et al., 1977).

The AChR-induced myasthenia in monkeys is particularly similar to the human disease in its clinical signs, including difficulties in breathing and swallowing, ptosis, motor weakness, etc., all being reversed temporarily following intra-

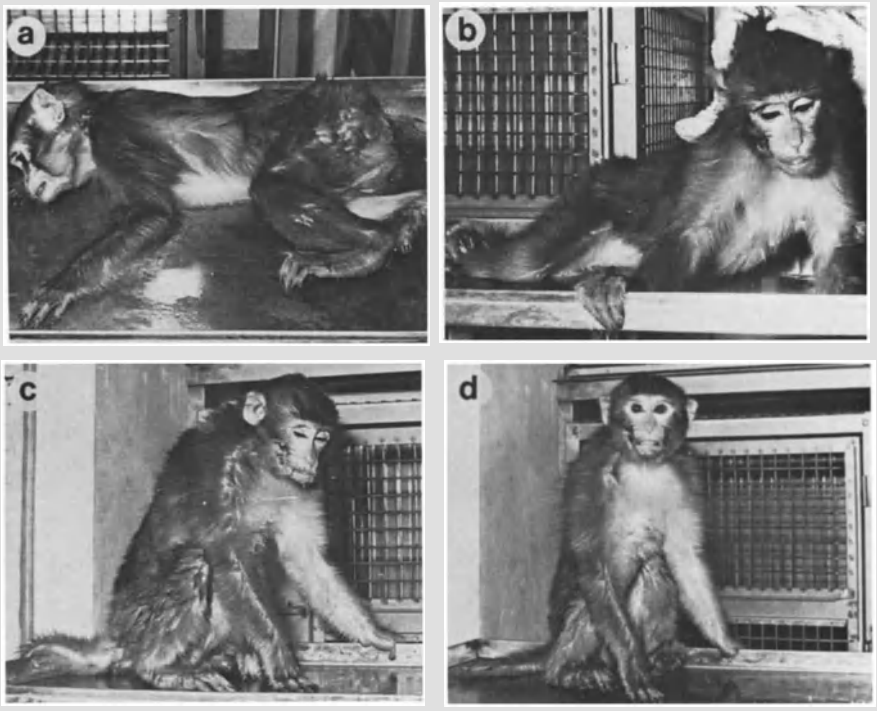

Fig. 6, *a–d*. Myasthenic monkey. *a and b* Before injection of Prostigmin. *c* 5 min after injection of Prostigmin. *d* 10 min after injection of Prostigmin (*Tarrab-Hazdai* et al., 1975c)

muscular injection of the anticholinesterase Prostigmin (Fig. 6) (*Tarrab-Hazdai* et al., 1975c).

An experimental model disease in mice seems of major importance, especially for studying genetic aspects of myasthenia gravis and the role of the thymus both as a specific antigenic target in the disease and as a source for immunocompetent helper and suppressor cells. The induction of the disease in mice is more difficult than its induction in other species so far studies. We succeeded in inducing EAMG in several inbred mouse strains following two immunizations with *Torpedo californica* AChR in a 9-week interval (*Fuchs* et al., 1976). EAMG was also induced in BALB/C mice with AChR purified from denervated rat muscle (*Granato* et al., 1976) and from syngeneic mouse denervated muscle (*Fulpius*, personal communication).

Sick mice suffered from weight loss and exhibited signs of fatigue, hypoactivity, ruffled fur, paralysis of the limbs, and motor impairment which were accentuated upon exercise. Their heads drooped and their backs were exaggeratedly humped (Fig. 7a). Severely sick animals died from the disease whereas in some of the mice the disease seemed to be transient. The clinical symptoms of mice with EAMG were temporarily reversed shortly after intravenous injection of 5 μg edrophonium chloride (Tensilon) (Fig. 7b). In particular motor performance, such as gripping and walking, showed improvement.

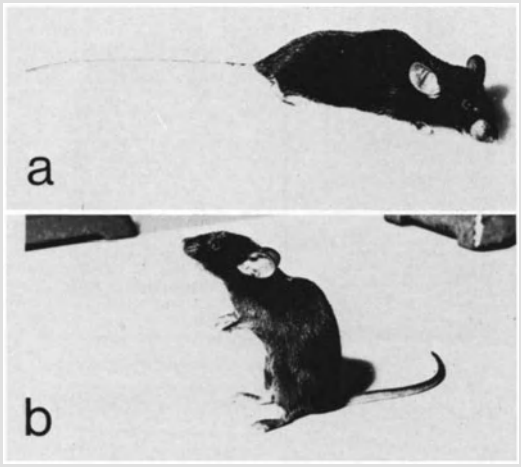

Fig. 7, *a and b*. Myasthenic C57BL/6 mouse 2 weeks after second injection of AChR. *a* Before Tensilon test. *b* 5 min after intravenous injection of Tensilon (*Fuchs* et al., 1978)

Cellular and humoral immune responses in the injected mice were determined. The autoimmune response against self AChR in C57BL/6 mice injected with AChR was demonstrated by reactivity to syngeneic muscle extracts. Cellular sensitivity was tested by the in vitro lymphocyte-transformation technique. Lymphocytes of *Torpedo* AChR-injected mice were stimulated in vitro when incubated with the immunogen, as well as with xenogeneic (rabbit) and syngeneic (C57BL/6) muscle extracts. Humoral autoimmune response was demonstrated by the ability of mouse anti-AChR sera to bind to syngeneic AChR labeled with ^{125}I-bungarotoxin (*Fuchs* et al., 1976). However, the humoral cross reactivity with syngeneic AChR should be interpreted with caution and by itself may not be a sufficient criterion for EAMG since similar cross reactivity was also observed in mice with high titers of antibodies to *Torpedo* AChR which exhibited no clinical signs of EAMG.

In recent experiments we tried to induce EAMG in mice given low dose irradiation (350 rad) or various doses of cyclophosphamide (20–200 mg/kg) before AChR administration. Low-dose cyclophosphamide (20 mg/kg) was given in order to selectively eliminate suppressor T cells (*Otterness* and *Chang*, 1976). In some experiments mice injected with low doses of cyclophosphamide had EAMG in a higher incidence than control AChR-injected mice without cyclophosphamide. It seems that B-cell function as well as helper T-cell function were not affected by low-dose cyclophosphamide treatment since the antibody titers of these mice were at least as high as those of AChR-injected mice with no drug treatment.

High-dose cyclophosphamide (150–200 mg/kg) administered to mice 2 days before injection with AChR resulted in the onset of EAMG in a high percentage of C57BL/6 mice 5–7 weeks after the receptor injection. EAMG was shown to be associated with intact cellular immune response as measured by delayed type hypersensitivity (ear test) whereas the humoral immune response was mark-

edly reduced (*Schwartz, Novick, Eshhar* and *Fuchs,* unpublished data). It may be that by varying the dose of cyclophosphamide it would be possible to selectively achieve cellular or humoral responses to AChR as was reported for other antigenic systems (*Ramshaw* et al., 1976, 1977). Such a study may contribute to the understanding of the mechanisms responsible for the induction of myasthenia as well as to the elucidation of the role of humoral vs. cellular mechanisms in the pathogenesis of this disease.

The clinical, physiologic, pharmacologic, and immunologic findings in animals with EAMG closely parallel the observed manifestations of MG. Both humoral and cellular immune responses to the immunizing electric organ AChR as well as to self AChR are observed in animals with EAMG.

B. The Immunologic Mechanism

The exact mechanism involved in the induction and maintenance of the experimental disease is not fully identified. Several immunologic factors were shown to participate in various stages of the disease. It appears that both cellular and humoral immune responses play a role in the pathogenesis of the blockade although cellular factors probably play the major role in the initial stages of the attack at the neuromuscular junction. Passive transfer of EAMG was achieved by transfering lymph node cells from donor guinea pigs immunized with purified AChR from *Torpedo californica*. Recipient animals revealed the same clinical signs and electromyographic patterns observed in actively challenged animals (*Tarrab-Hazdai* et al., 1975b). Similar results were also reported by *Lennon* et al. (1976) for rats. We did not succeed in transfering EAMG to rabbits or guinea pigs by means of syngeneic immunoglobulins or purified antibodies of myasthenic animals. However, passive transfer of EAMG was observed in mice following a transfer of IgG from myasthenic patients (*Toyka* et al., 1977). The efficacy of the transfer was found by these authors to be partially dependent on complement C_3. EAMG was passively transfered in rats following an intravenous injection of serum or IgG from a rat with chronic EAMG (*Lindstrom* et al., 1976b).

Anti-AChR antibodies as well as immunoglobulins from myasthenic patients were shown to block the binding of α-bungarotoxin to AChR in in vitro muscle cell cultures, to accelerate the degradation of AChR and reduce acetylcholine sensitivity (*Kao* and *Drachman*, 1977a; *Appel* et al., 1977; *Anwyl* et al., 1977; *Bevan* et al., 1977; *Heinemann* et al., 1977; *Prives* et al., 1978). These reports suggest that a similar mechanism may possibly be involved in the autoimmune pathogenesis of myasthenia gravis and that the effect of antibodies in vivo might result from direct binding to AChR at the neuromuscular junction and inducing decreased AChR content. Recent data (*Lennon* et al., 1978) suggest that complement is a mediator of the pathogenic effect of anti-AChR antibodies in vivo and that the in vitro effects of antibodies on the accelerated degradation and impairment of the ionophore function of AChR do not play a significant role in vivo in impairing neuromuscular transmission in an intact neuromuscular junction.

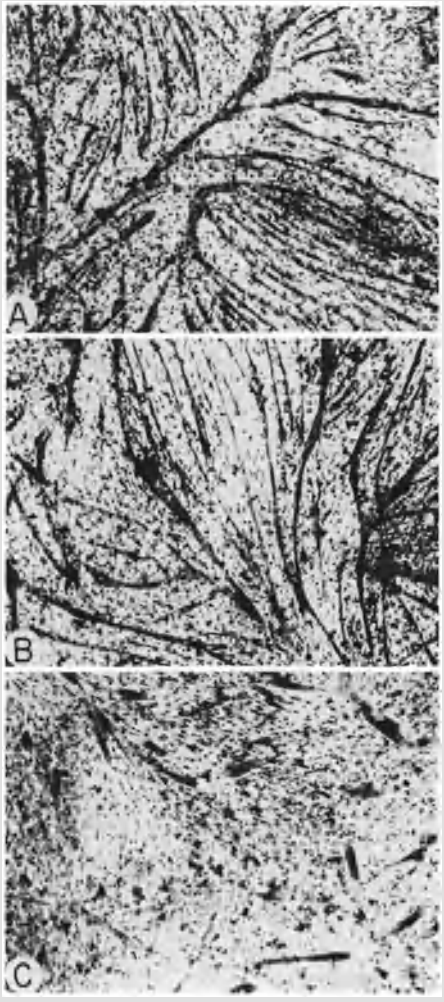

Fig. 8, *a–c*. Destructive effect of macrophages from AChR-sensitized rats on muscle cell cultures. 48-h-old rat muscle cell cultures grown in a medium promoting cell proliferation without fusion were transfered to fusion-permissive S-medium (*Yaffe*, 1973) containing rat macrophages (4×10^6/plate). (A) Macrophages of normal rats. (B) Macrophages of CFA-injected rats. (C) Macrophages of myasthenic rats. Fixed 72 h after addition of macrophages. Giemsa stain; $\times 4$

Cytophilic antibodies were shown to play a role in the pathogenesis of EAMG. We have demonstrated that macrophage cytophilic antibodies with AChR specificity are present in rabbits with EAMG (*Martinez* et al., 1977). Cytophilic antibodies capable of binding to normal alveolar macrophages are detected in the sera of rabbits 14 days after immunization with AChR and are maintained through the severe stages of the disease. In addition cytophilic anti-AChR antibodies are bound in vivo to macrophages of sick rabbits, as

measured by the direct binding of ^{125}I-AChR to alveolar macrophages drawn from sick rabbits.

The role of cytophilic antibodies in EAMG has not yet been established; they may bind first to the AChR in the target tissue and only then bind locally to macrophages via their Fc receptors. Another possibility is that cytophilic antibodies bind first to macrophages with high avidity for the specific antibodies and only then are the antibody-coated cells specifically directed to the antigenic target tissue. Since we have observed antibody-coated macrophages in the alveolar fluid of sick animals, not associated with the synaptic membrane, and since *Engel* et al. (1976) have reported on the invasion of macrophages in the region of the damaged end plate, we propose that antibody-coated macrophages reach the target tissue (*Martinez* et al., 1977).

The involvement of macrophages in the pathogenesis of myasthenia was studied recently by utilizing an in vitro system of primary cultures of skeletal muscle cells (*Tarrab-Hazdai* et al., 1978 b). Macrophages from myasthenic AChR-sensitized rats of from either normal rats or rats injected with Freund's complete adjuvant were added to rat skeletal muscle cell cultures before or after cell fusion. Macrophages of myasthenic rats had a drastic morphologic effect on the cultures. When macrophages were added before cell fusion, the cultures formed a much smaller number of fibers, most of them short and abnormal (Fig. 8). Addition of such macrophages after cell fusion also resulted in partial destruction of the fibers. The cytotoxic effect of the macrophages was verified by decreased levels of creatine kinase and protein synthesis. The effect of the macrophages was specific to muscle cells since no toxic effect of the macrophages on other cell types, such as kidney cells, was observed (*Tarrab-Hazdai* et al., 1978 b). It is of interest that antibodies or lymphocytes from AChR-sensitized rats had no destructive effect on the muscle cell cultures. The cytotoxic effect of macrophages on muscle cells suggests that the macrophages react against antigenic determinants involved in muscle differentiation.

Studies on the effect of immunosuppressive drugs on EAMG as well as immunologic analysis of the receptor molecule and its distinct antigenic determinants are also helpful for elucidation of the molecular and immunologic mechanisms involved in EAMG. Such studies are described in other sections of this review.

C. The Role of the Thymus

A model involving an autoimmune response to AChR seems to explain adequately many clinical and physiologic manifestations of myasthenia gravis. However, any general model for the pathogenesis of MG must also take into account the involvement of the thymus in this condition. A high incidence of thymic hyperplasia or neoplasia is found in patients with MG and thymectomy is beneficial in the management of some patients. Immunologic studies of myasthenic patients have demonstrated the presence of humoral and cellular immune responses towards thymic tissues. *Goldstein* (1968) and *Kalden* et al. (1969) have shown that animals immunized with thymic extracts develop an autoimmune thymitis as well as a partial defect in neuromuscular transmission.

The relation between the thymic disorder and the neuromuscular block is not yet understood. An autoimmune response, resulting from antigenic alterations, may cause damage to both neuromuscular junctions and to the thymus. They may both be simultaneously involved or one of them may be affected primarily and the other secondarily due to immunologic cross reaction between them. In view of these possibilities we looked for immunologic cross reaction between AChR and thymus and have shown that electric organ AChR cross reacts with components of calf thymus, both on the cellular and humoral levels of the immune response (*Aharonov* et al., 1975c). Such a cross reaction can provide a molecular basis for the association between neuromuscular block and thymic disorders in MG.

The origin of the cross reaction between thymus and AChR may stem from myoid cells in the thymus. Cultures of thymus cells from mice, rats, and humans were recently shown to yield skeletal muscle colonies possessing demonstrable amounts of AChR on their cell membranes (*Wekerle* et al., 1975; *Kao* and *Drachman*, 1977b). In other studies it was observed that α-bungarotoxin binds to epithelial cells of thymus (*Engel* et al., 1977). It is possible that such cells in the thymus play a role in the pathogenesis of myasthenia gravis. Alternatively, antigenic markers cross reactive with antigenic determinants of AChR may be present on thymus cells. Preliminary results from our laboratory (unpublished data) using immunofluorescent techniques and anti-AChR antibodies support this possibility.

AChR is a thymus-dependent antigen. We have shown by adoptive transfer experiments that the humoral response against AChR is T-cell dependent (*Fuchs* et al., 1976). Similar results were also obtained in rats (*Lennon* et al., 1976). EAMG in neonatally thymectomized rabbits was suppressed without alteration of antibody titers, also suggesting the role of T cells in the pathogenesis of myasthenia (*Penn* et al., 1977).

D. Genetic Aspects

Susceptibility to myasthenia gravis in humans is genetically controlled by and associated with the major histocompatibility antigens (HL-A) (*Fritze* et al., 1976; *Pirskanen*, 1976; *Oosterhuis* et al., 1976). EAMG in mice and the availability of many inbred mouse strains provide a valuable tool for studying genetic control of the immune and autoimmune response to AChR. We have demonstrated different susceptibility to EAMG in strains representing different haplotypes of the major histocompatibility complex (H-2) (*Fuchs* et al., 1976). Mice were injected twice in a 9-week interval with 10 μg purified *Torpedo californica* AChR. Clinical signs of EAMG were observed in mice of inbred strains with $H\text{-}2^a$, $H\text{-}2^b$, $H\text{-}2^d$, and $H\text{-}2^k$ haplotypes. The disease was not found in any mice carrying $H\text{-}2^q$ or $H\text{-}2^s$ haplotypes. It is noteworthy that no correlation between the antibody titers to *Torpedo* AChR and incidence of the disease was found, for mice of all strains tested gave similarly high titers. It is possible, however, that there are differences in the specificity of the antibodies. The susceptibility to EAMG may be determined by a genetically controlled ability

to respond to a specific determinant or determinants. It was recently proposed that aside from the possibility that the autoimmune responses to AChR are under genetic control, the capacity of mouse thymic stem cells to differentiate into myogenic cells in vitro is hereditary sex dependent, and associated with the major histocompatibility antigens (*Wekerle* and *Ketelsen*, 1977).

E. Regulation of EAMG as a Therapeutic Approach

Aside from the use of EAMG as a tool for studying the pathogenesis of MG it is also valuable for investigating the mechanism of action and optimal regimes of different drugs used in therapy of MG. Moreover, the availability of an experimental model disease induced by a well-characterized antigen (AChR) enables us to attempt the development of specific immunotherapy for MG. We have used the disease in rabbits to achieve suppression of EAMG by nonspecific immunosuppressive drugs and by an nonpathogenic derivative of the AChR molecule. An additional approach for specific regulation of EAMG is attempted in the preparation and application of anti-idiotypic antibodies specific to AChR idiotypes.

Nonspecific immunosuppressive drugs: Corticosteroids and the antimetabolite azathioprine were empirically found to be effective in treatment of MG (*Jenkins*, 1972; *Warmolts* and *Engel*, 1972; *Seybold* and *Drachman*, 1974; *Matell* et al., 1976). We have used the same drugs to suppress EAMG in rabbits and to follow the mechanism of suppression. Rabbits injected with purified *Torpedo californica* AChR were treated with hydrocortisone according to one of two regimes: either early continuous administration of high doses of hydrocortisone or administration of gradually increasing doses of hydrocortisone (*Abramsky* et al., 1976). The effect of corticosteroid treatment seemed to depend on the regimen. When hydrocortisone was administered in high doses from the beginning, EAMG appeared earlier and in a more severe form than in the control animals. Nevertheless, this treatment had some suppressive effect. The schedule of steroid administration also seems of great importance for minimizing side effects in MG patients; in spite of the overall beneficial effect of steroids in MG, increasing weakness usually occurs early in treatment and can be avoided by a gradually increasing dosage schedule.

The effect of hydrocortisone in suppressing EAMG was paralleled by a diminished cellular sensitization to AChR in vitro (*Abramsky* et al., 1976). This is in agreement with our findings that in human myasthenia decreased cellular sensitization to AChR correlated with clinical improvement during prednisone therapy (*Abramsky* et al., 1975a).

We have shown that azathioprine (Az) is effective in suppressing the onset of EAMG in rabbits injected with AChR from *Torpedo californica* and have studied the correlation between clinical effects and several immunologic parameters resulting from this immunosuppressive treatment (*Abramsky* et al., 1976; *Tarrab-Hazdai* et al., 1977a, b).

Rabbits were injected on day 1 with AChR (80 μg, in Freund's complete adjuvant intradermally) and with Az (4 mg/kg, intramuscularly). Similar admin-

Table 1. Suppression of AChR-induced EAMG by azathioprine

Treatment[a]	EAMG			Surviving after 12 months (%)
	Onset (days)	Clinical signs (%)	Death (%)	
Saline (control)	21–30	100(8/8)[b]	100(8/8)	0
Azathioprine (5 months)	100	10(1/10)	20(2/10)[c]	80(8/10)

[a] All rabbits were injected with 80 μg *Torpedo californía* AChR on day 1.
[b] The numbers in parenthesis represent the number of animals.
[c] No clinical signs of EAMG were detected in one of these rabbits

istrations of Az were given to the rabbits daily for 15 days and then every 2–3 days for an additional 5 months. In a control group the Az injections were replaced by saline injections.

The Az treatment effectively suppressed the onset of EAMG in rabbits, for at least 12 months, even after discontinuing the drug treatment for 7 months (Table 1) (*Tarrab-Hazdai* et al., 1977a). A second injection of AChR 12 months later to such immunosuppressed rabbits led to the onset of EAMG after a further 6–10 days, as is the case after a secondary injection with AChR. This behavior suggests that although EAMG was prevented, immunologic memory was maintained (*Tarrab-Hazdai* et al., 1977b).

The suppressive effect of Az was accompanied by decreased cellular and humoral immunologic reactivity against both the immunizing *Torpedo* AChR and self AChR, with a significantly more pronounced effect on the response to self receptor (*Tarrab-Hazdai* et al., 1977a). Recent studies have demonstrated that the preventive effect of Az on EAMG and the mechanism of immunosuppression seem to correlate also with the dose of drug applied. Complete prevention of EAMG was achieved upon administration of high doses of Az (10 mg/kg). Significant decrease of both humoral and cellular immune responses was observed, as also noticed with low dose Az treatment (4 mg/kg).

Az treatment resulted in qualitative changes in the antibody class, specificity, and affinity (*Tarrab-Hazdai* et al., 1977b). Antisera from immunosuppressed animals treated with high doses of Az were more sensitive to β-mercaptoethanol treatment than sera of untreated control rabbits, suggesting that the effect of Az was caused by selectively inhibiting IgG or, alternatively, by inhibiting the shift from IgM to IgG production.

The effect of Az treatment on the specificity of the antibodies was tested by comparing the ability of the different antisera to bind native AChR with the ability to bind RCM-AChR, a nonmyasthenic cross-reactive derivative of the receptor (*Bartfeld* and *Fuchs*, 1977). Figure 9 shows a difference in the relative binding of AChR and RCM-AChR between sera from Az-treated and nontreated animals. In the sera of Az-treated groups both AChR and RCM-

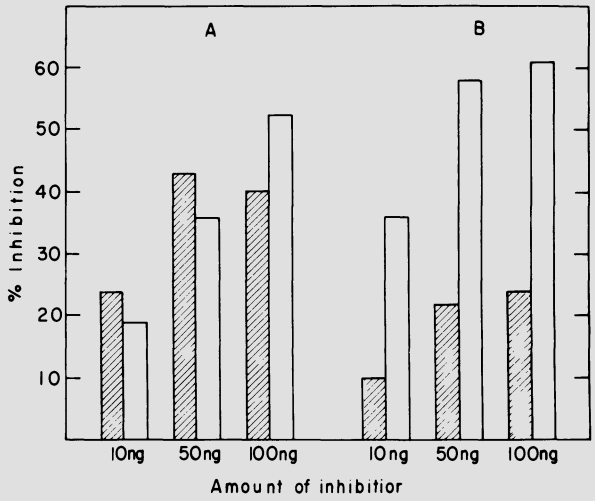

Fig. 9, *a and b*. Inhibition of the binding of ^{125}I-AChR to rabbit antisera by AChR (*white areas*) and by RCM-AChR (*shaded areas*). (A) AChR-injected rabbits treated with Az. (B) AChR-injected rabbits (nontreated control)

AChR inhibited to a similar extent the binding to the antibodies (Fig. 9a). Such a pattern of inhibition was also observed in *nonmyasthenic* rabbits injected repeatedly with RCM-AChR (*Bartfeld* and *Fuchs*, 1977; *Fuchs* et al., 1978). In sera from the control group of AChR-injected rabbits (not treated with Az) AChR was a better inhibitor than RCM-AChR (Fig. 9b), demonstrating that these antisera contained antibodies against determinants specific to the native receptor which are not present in RCM-AChR. These results suggested that in addition to suppressing the level of total serum antibody, Az selectively affected antibodies against certain antigenic determinants that may be specifically involved in the myasthenic activity of AChR (*Tarrab-Hazdai, Schwartz* and *Fuchs*, unpublished results).

The association between the immunosuppressive effect of Az on AChR-induced EAMG and the affinity of the antibodies was studied, utilizing purified anti-AChR antibodies (*Schwartz* et al., 1978a). Mathematical analysis of the data obtained from binding experiments of ^{125}I-AChR to purified antibodies suggested that treatment with Az decreased the amount of anti-AChR antibodies possessing high-affinity values (*Schwartz* et al., 1978a).

The immunosuppressive treatment of EAMG provides a valuable tool for elucidating many aspects of the mechanism of action of nonspecific immunosuppressive drugs in the therapy of autoimmune diseases in gernal and, in particular, for establishing the optimal conditions for Az therapy in MG.

Specific immunosuppression of EAMG: Although immunosuppressive drugs seem useful in treating both the human and experimental diseases, such agents may suppress the whole immune system and exert nonspecific toxic effects. It is obvious that the treatment of choice, if possible, should be a *specific* one, namely a drug which will affect selectively the immunologic reactivity

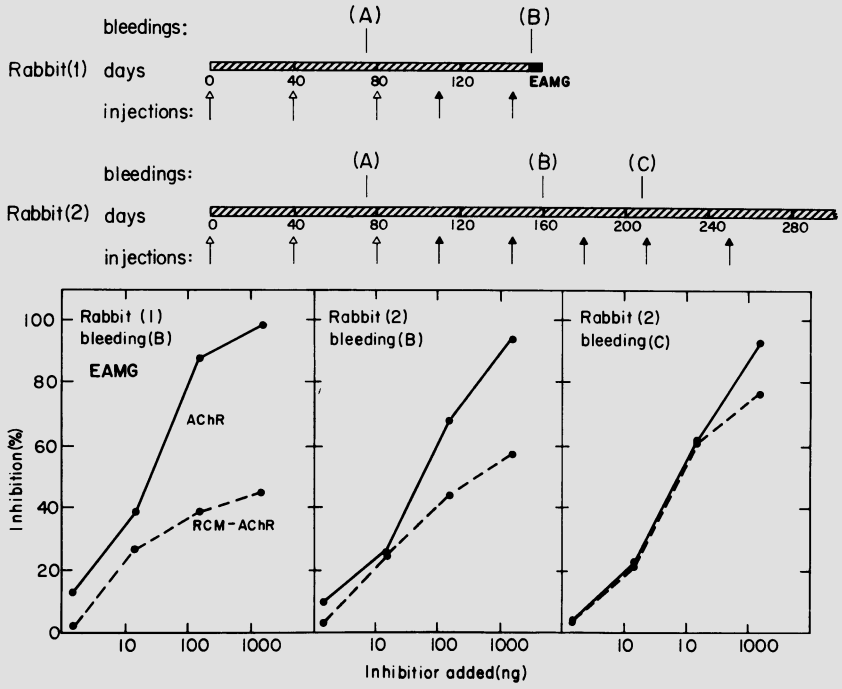

Fig. 10. Effect of preimmunization with RCM-AChR on the onset of EAMG and on the antibody specificity. Inhibition of the binding of ^{125}I-AChR to sera from sick (*1*) and protected (*2*) rabbits by AChR (——) and RCM-AChR (– – –). The immunization course, the bleedings, and the development of EAMG are described schematically at the top of the fiature. *Open arrows* (↑) represent injection with RCM-AChR and *closed arrows* (↑) represent injection with AChR (*Bartfeld* and *Fuchs*, 1978)

leading to the neuromuscular disorder but leave the overall immune response intact. With a well-defined antigen (AChR) that induces EAMG and is the autoantigen in MG, one may try by molecular modifications and immunologic analysis to design and achieve such a specific immunosuppressive derivative.

In the earlier part of this chapter I reviewed the immunochemical analysis of several derivatives of AChR prepared from native AChR by several modification procedures. Since denatured AChR (i.e., reduced carboxymethylated AChR, RCM-AChR (*Bartfeld* and *Fuchs*, 1977)) did not by itself induce any EAMG symptoms and cross reacted with AChR it seemed an appropriate candidate for attempting specific immunosuppression of EAMG. Indeed, RCM-AChR was shown to be capable of both preventing the onset of EAMG and of suppressing the disease in myasthenic rabbits (*Fuchs* et al., 1978; *Bartfeld* and *Fuchs*, 1978). For the protection experiments rabbits were preimmunized two or three times with RCM-AChR and were then injected with the intact receptor. Under these conditions the onset of EAMG was either delayed or completely prevented in rabbits preimmunized with RCM-AChR. EAMG was prevented for at least 10 months even when four or five injections of AChR were administered during that period (Fig. 10). A delay in the onset of EAMG was usually observed

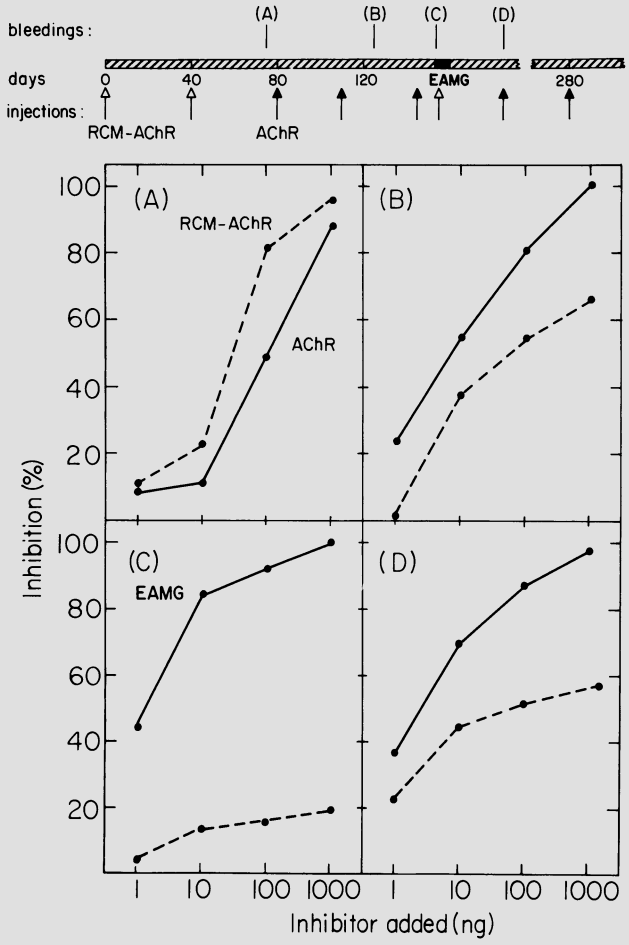

Fig. 11, *a–d*. Correlation between the therapeutic effect of RCM-AChR and the antigenic specificity of the antibodies. Bleedings (A) and (B) before the onset of EAMG; Bleeding (C) during clinical signs of EAMG; Bleeding (D) after the rabbit was cured following RCM-AChR injection. The inhibition of the binding of ^{125}I-AChR to the rabbit's serum by AChR (—) and RCM-AChR (– – –) was measured for each bleeding. The immunization course, the bleedings, and the development of EAMG are described schematically at the *top* of the figure (*Bartfeld* and *Fuchs*, 1978)

in rabbits given only two injections of RCM-AChR prior to immunization with AChR.

In addition to the protective potential of RCM-AChR, its therapeutic effect on myasthenic rabbits was observed as well. A single administration of RCM-AChR in Freund's complete adjuvant to myasthenic rabbits during the initial stages of clinical development of EAMG led to a complete suppression of EAMG in at least 10 out of 20 myasthenic rabbits (Figs. 11 and 12) (*Fuchs* et al., 1978; *Bartfeld* and *Fuchs*, 1978).

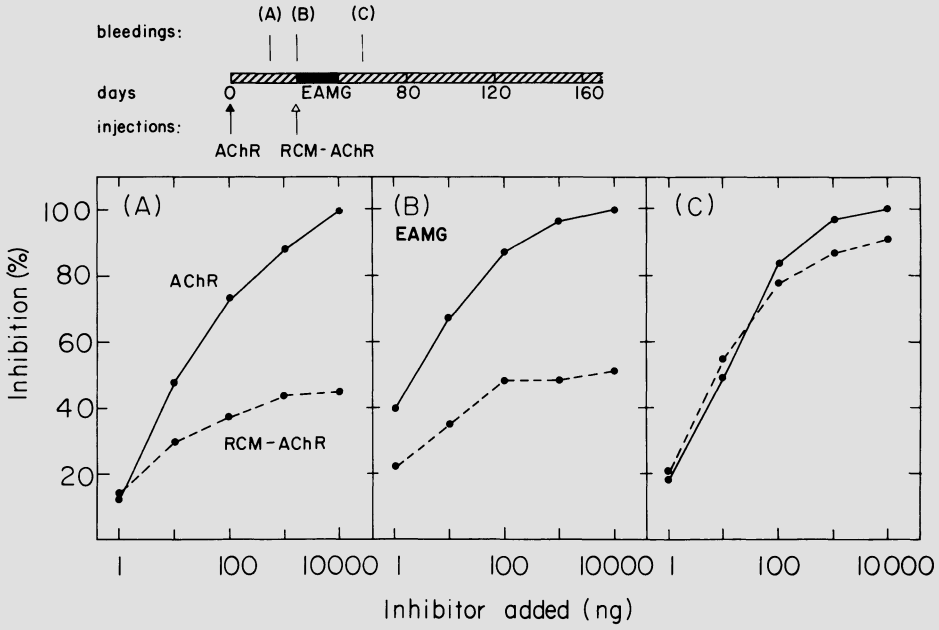

Fig. 12. Therapy of EAMG by RCM-AChR. Correlation between the clinical symptoms and the antigenic specificity of the antibodies in a representative myasthenic rabbit in which the disease had been reversed by treatment with RCM-AChR. The inhibition of the binding of ^{125}I-AChR to the rabbit's serum by AChR (——) and RCM-AChR (– – –) was measured for bleedings A, B and C. The course of immunizations, bleeding, and development of EAMG are described schematically at the *top* of the figure (*Bartfeld* and *Fuchs*, 1978)

There seemed to be a correlation between the clinical conditions of the RCM-AChR treated rabbits and the antigenic specificity of their immune response. Differences both in antibody titers and antigenic specifity were ascertained between sera of rabbits in which EAMG was prevented or reversed by RCM-AChR and those of sick, nontreated rabbits (Figs. 10, 11 and 12) (*Fuchs* et al., 1978; *Bartfeld* and *Fuchs*, 1978).

The mechanism of the therapeutic effect of RCM-AChR is not fully understood as yet. The cross reactivity between AChR and RCM-AChR and the nonpathogenecity of the latter appear to be crucial in governing the immunosuppressive and therapeutic effects of RCM-AChR on EAMG. It is possible that the humoral responses accompanying the prevention, appearance, or suppression of EAMG represent relative levels of two different antibody populations against AChR, one of which is specific to a myasthenic determinant in the AChR molecule and is involved in the disease, whereas the other is directed to other antigenic determinants not involved in the disease. AChR can elicit antibodies to both types of determinants and it is the immune response against the myasthenic determinants which is responsible for induction of EAMG. RCM-AChR elicits an immune response to determinants other than the myasthenic ones and is, therefore, not myasthenic by itself. However, anti-RCM-AChR anti-

bodies can bind to the native receptor (*Bartfeld* and *Fuchs*, 1977) and probably also to the self receptor and this may be the basis for the preventive and therapeutic effect of RCM-AChR. Also, antibodies to the myasthenic determinants may be suppressed by a mechanism of antigenic competition following RCM-AChR injection. The therapeutic effect of RCM-AChR may thus be explained by high levels of antibodies to the nonmyasthenic determinants which do not block the physiologic function of the receptor and compete with antibodies against the myasthenic determinants on the binding to self receptor. RCM-AChR may also bind in vivo to anti-AChR antibodies or to AChR-sensitized cells and neutralize their pathologic effects (*Bartfeld* and *Fuchs*, 1978).

The potential cure of myasthenic rabbits by nonmyasthenic derivatives of AChR may have practical implications. Additional chemical modifications of AChR and its further enzymic or chemical degradation may yield more information concerning the molecular basis of the autoimmune response to AChR. We have shown recently that poly-DL-alanyl AChR is nonmyasthenic in rabbits (*Schmidt-Sole* et al., 1977) and are currently testing its possible therapeutic potential.

Anti-idiotypes: Anti-idiotypes may have a regulatory role in autoimmune diseases or other disorders of an immunologic nature. The application of anti-idiotypic antibodies furnishes a possible approach for specific regulation of the immune response to AChR. Anti-idiotypic serum specific to anti-AChR idiotypes was prepared in C57BL/6J mice by repeated injections with purified C57BL/6J anti-AChR antibodies or with syngeneic spleen cells educated with AChR (*Schwartz* et al., 1978 b). The anti-idiotypic serum reacted specifically with anti-AChR antibodies of several mouse strains and of other species. The anti-idiotypic serum was also able to inhibit the binding of ^{125}I-AChR to mouse anti-AChR antibodies, suggesting that idiotypic determinant(s) against which the serum is directed is (are) associated with the antigen-combining site (*Schwartz* et al., 1978 b).

The broad cross reactivity among anti-AChR idiotypic determinants of different mouse strains as well as those of different species suggest that similar idiotypic determinants exist in the anti-AChR antibodies tested. Such a situation could be explained if all the tested species inherited a set of immunoglobulin variable region (V_H) genes which retained part of the hypervariable region sequence necessary to produce anti-AChR antibodies. This kind of inheritance could be expected in case of an evolutionary, highly conserved antigen like AChR.

V. Autoimmune Response to AChR in Myasthenia Gravis

As previously mentioned, the possibility that myasthenia gravis is an autoimmune disease has been prominent since the early works of *Simpson* (1960) and of *Nastuk* et al. (1960). The association of MG with other autoimmune diseases, the occurrence of thymic abnormalities in a high percentage of myasthenics, and the beneficial effects of thymectomy and steroid therapy supplied clinical evidence for this possibility. Immunologic studies have demonstrated both lymphocyte-mediated immunologic reactions as well as circulating antibo-

dies to muscle and thymic antigens in myasthenic patients. In view of this circumstantial evidence and together with the findings on decreased postsynaptic AChR in MG (*Fambrough* et al., 1973) and the induction of EAMG in animals following immunization with purified AChR, an autoimmune response to AChR in myasthenia gravis was sought. Indeed, in the last 4 years many reports have indicated the presence of both circulating antibodies and sensitized lymphocytes specific to AChR in MG and their involvement in the pathogenesis of myasthenia.

Circulating antibodies specific to AChR were observed in a high percentage of patients with MG (*Aharonov* et al., 1975a; *Bender* et al., 1975; *Appel* et al., 1975; *Lindstrom* et al., 1976c). The level of circulating antibody estimated depends very much on the assay used and on the cross reactivity between the test antigen and human AChR. Using the microcomplement fixation assay with purified *Torpedo* AChR as the antigen we have observed antibodies to AChR in about 80% of the patients tested (*Aharonov* et al., 1975a). With a radioimmunoassay and a preparation of nonpurified human muscle AChR significant titers of antibodies against human AChR were found in more than 90% of the patients studied (*Lindstrom* et al., 1976c). We observe similar titers in some myasthenic patients when testing their sera by a radioimmunoassay with muscle AChR preparations obtained from denervated rats and labeled specifically with ^{125}I-α-bungarotoxin (*Brener* et al., 1978). This assay is similar to the one developed by *Appel* et al. (1975).

The anti-AChR antibody activity of immunoglobulins from myasthenic patients was verified by their accelerating effect on the degradation of AChR in tissue culture (*Kao* and *Drachman*, 1977a; *Anwyl* et al., 1977; *Bevan* et al., 1977). Whether this in vitro effect of the immunoglubulin is also involved in the pathogenesis of myasthenia in vivo is still an open question.

There is increasing evidence that circulating anti-AChR antibodies may be actively involved in causing the receptor abnormality. Myasthenic symptoms have been produced in mice following a passive transfer of high amounts of immunoglobulin fraction from the serum of myasthenic patients (*Toyka* et al., 1977). The beneficial effect of lymph drainage (*Matell* et al., 1976) and of plasma exchange (*Pinching* et al., 1976; *Dau* et al., 1977) in myasthenic patients also supports the direct role of antibodies in myasthenia. Plasma exchange removes humoral factor from the blood and produces a short-term remission. An inverse relationship between anti-AChR titer and muscle strength (*Newsom Davis* et al., 1978) follows plasma exchange.

Cell-mediated immunity to AChR is observed in myasthenic patients. Peripheral blood lymphocytes from patients with MG were shown to be stimulated when cultured in vitro with an electric eel extract enriched with AChR (*Abramsky* et al., 1975b). These findings suggest that an in vivo sensitization of lymphocytes to self AChR occurs in MG and that the cell-mediated autoimmune mechanism may be important in the pathogenesis of the neuromuscular block. Similar results were reported later using purified *Torpedo* AChR for stimulating the lymphocytes in vitro (*Richman* et al., 1976).

The lymphocyte response to AChR did not seem to correlate with either the clinical condition or the time lapse between onset of the illness and the

test. However, there seems to be a correlation between the stimulation indexes obtained in the in vitro lymphocyte-transformation technique and therapy. Higher stimulation indexes were observed in patients who had undergone neither steroid therapy nor thymectomy, whereas lower values were obtained in patients following either of these treatments (*Abramsky* et al., 1975b). Marked diminuation of the cellular response to AChR was shown in patients who displayed clinical improvement after prednisone treatment (*Abramsky* et al., 1975a).

VI. Concluding Remarks

The nicotinic receptor for acetylcholine provides a unique model system encompassing a wide range of disciplines from the more theoretical questions in membrane receptor research down to the origin and therapy of a grave human disease, myastenia gravis. The investigation of AChR has made marked progress in several directions in a brief period of time. In the present review I have focused on immunologic aspects of AChR. This topic has been extensively investigated during the past 5 years. As a result of these studies it is now evident that myasthenia gravis is an autoimmune disease in which AChR is a major autoantigen. Experimental myasthenia is induced by immunization with AChR and is an appropriate system for studying the origin, mechanism, and therapy of the human disease. Progress in the structural and physiologic analysis of AChR is leading to the development of new immunologic approaches. It is now possible to achieve specific immunosuppression of experimental myasthenia by utilizing nonpathogenic derivatives of the receptor molecule. Hopefully this approach will contribute in the future to treatment of the human disease.

Antibodies to AChR and to distinct smaller regions of this molecule are being used as tools for studying about the evolution, development, and spatial distribution of AChR in the excitable membrane, as well as the correlation of structural features of the receptor and its biologic function.

The investigation of the immunologic properties of AChR has been very rewarding from both biologic and medical points of view. Moreover, this study may have a broader significance for the general understanding of autoimmune phenomena involving membrane receptors as antigens.

Acknowledgements. I wish to thank my colleagues, A. Amsterdam, J. Prives, R. Tarrab-Hazdai, M. Schwartz, and D. Novick for helpful discussions and suggestions during the preparation of the manuscript. Part of the work from our laboratory cited in this review was supported by grants from the Los Angeles Chapter of the Myasthenia Gravis Foundation and from the United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel.

References

Abramsky, O., Aharonov, A., Teitelbaum, D., Fuchs, S.: Myasthenia gravis and acetylcholine receptor: Effect of steroids in clinical course and cellular immune response to acetylcholine receptor. Arch. Neurol. *32*, 684–687 (1975a)

Abramsky, O., Aharonov, A., Webb, C., Fuchs, S.: Cellular immune response to acetylcholine receptor-rich fraction in patients with myasthenia gravis. Clin. Exp. Immunol. *19*, 11–16 (1975b)

Abramsky, O., Tarrab-Hazdai, R., Aharonov, A., Fuchs, S.: Immunosuppression of experimental autoimmune myasthenia gravis by hydrocortisone and azathioprine. J. Immunol. *117*, 225–228 (1976)

Aharonov, A., Abramsky, O., Tarrab-Hazdai, R., Fuchs, S.: Humoral antibodies to acetylcholine receptor in patients with myasthenia gravis. Lancet *1975a/II*, 340–342

Aharonov, A., Kalderon, N., Silman, I., Fuchs, S.: Preparation and immunochemical characterization of a water-soluble acetylcholine receptor fraction from the electric organ tissue of the electric eel. Immunochemistry *12*, 765–771 (1975b)

Aharonov, A., Tarrab-Hazdai, R., Abramsky, O., Fuchs, S.: Immunological relationship between acetylcholine receptor and thymus: a possible significance in myasthenia gravis. Proc. Natl. Acad. Sci. U.S.A. *72*, 1456–1459 (1975c)

Aharonov, A., Tarrab-Hazdai, R., Silman, I., Fuchs, S.: Immunochemical studies on acetylcholine receptor from *Torpedo californica*. Immunochemistry *14*, 129–137 (1977)

Anwyl, R., Appel, S.M., Narahashi, T.: Myasthenia gravis serum reduces acetylcholine sensitivity in cultured rat myotubes. Nature *267*, 262–263 (1977)

Appel, S.H., Almon, R.R., Levy, N.: Acetylcholine receptor antibodies in myasthenia gravis. N. Engl. J. Med. *293*, 760–761 (1975)

Appel, S.H., Anwyl, R., McAdams, M.W., Elias, S.: Accelerated degradation of acetylcholine receptor from cultured rat myotubes with myasthenia gravis sera and globins. Proc. Natl. Acad. Sci. U.S.A. *74*, 2130–2134 (1977)

Arnon, R., Maron, E.: An immunological approach to the structural relationship between hen egg-white lysozyme and bovine α-lactabumin. J. Mol. Biol. *61*, 225–235 (1971)

Bartfeld, D., Fuchs, S.: Immunological characterization of an irreversibly denatured acetylcholine receptor. F.E.B.S. Lett. *77*, 214–218 (1977)

Bartfeld, D., Fuchs, S.: Specific immunosuppression of experimental autoimmune myasthenia gravis by denatured acetylcholine receptor. Proc. Natl. Acad. Sci. U.S.A. *75*, 4006–4010 (1978)

Bender, A.N., Ringel, S.P., Engel, W.K., Daniels, M.P., Vogel, Z.: Myasthenia gravis: a serum factor blocking acetylcholine receptors of the human neuromuscular junction. Lancet *1975/I*, 607–609

Bevan, S., Heinemann, S., Lennon, V.A., Lindstrom, J.: Reduced muscle acetylcholine sensitivity in rats immunized with acetylcholine receptor. Nature *260*, 438–439 (1976)

Bevan, S., Kullberg, R.W., Heinemann, S.F.: Human myasthenic sera reduce acetylcholine sensitivity of human muscle cells in tissue culture. Nature *267*, 263–265 (1977)

Brener, T., Abramsky, O., Lisak, R.P., Zweiman, B., Tarrab-Hazdai, R., Fuchs, S.: Antibodies to acetylcholine receptor in serum of patients with myasthenia gravis: Radioimmunoassay. Isr. J. Med. Sci. *14*, 986–989 (1978)

Briley, M.S., Changex, J.-P.: Isolation and purification of the nicotinic acethylcholine receptor and its functional reconstitution into a membrane environment. Int. Rev. Neurobiol. *20*, 31–63 (1977)

Brockes, J.P., Hall, Z.W.: Synthesis of acetylcholine receptor by denervated rat diaphragm muscle. Proc. Natl. Acad. Sci. U.S.A. *72*, 1368–1372 (1975)

Changeux, J.-P.: The cholinergic receptor protein from fish electric organ. In: handbook of Pychopharmacology. *Iversen, L.L.* et al. (eds.) New York: Plenum Press 1975, Vol. VI, pp. 235–301

Claudio, T., Raftery, M.A.: Immunological comparison of acetylcholine receptors and their subunits from species of electric ray. Arch. Biochem. Biophys. *181*, 484–489 (1977)

Cohen, J.B., Weber, M., Huchet, M., Changeux, J.-P.: Purification from Torpedo marmorata electric tissue of membrane fragments particularly rich in cholinergic receptor. F.E.B.S. Lett. *26*, 43–47 (1972)

Dau, P.C., Lindstrom, J.M., Cassel, C.K., Denyl, E.H., Shev, E.E., Spitler, L.E.: Plasmapheresis and immunosuppressive drug therapy in myasthenia gravis. N. Engl. J. Med. *24*, 1134–1140 (1977)

Engel, A.G., Tsujihata, M., Lindstrom, J.M., Lennon, V.A.: The motor end plate in myasthe-

nia gravis and in experimental autoimmune myasthenia gravis. A quantitative ultrastructural study. Ann. N.Y. Acad. Sci. *274*, 60–79 (1976)

Engel, W.K., Trotter, J.L., McFarlin, D.E., McIntosh, C.L.: Thymic epithelial cell contains acetylcholine receptor. Lancet *1977/I*, 1310–1311

Fambrough, D.M., Drachman, D.B., Satyamurti, S.: Neuromuscular junction in myasthenia gravis: decreased acetylcholine receptors. Science *182*, 293–295 (1973)

Fritze, D., Herrmann, C., Jr., Naeim, F., Smith, G.S., Zeller, E., Walford, R.L.: The biologic significance of HL-A antigen markers in myasthenia gravis. Ann. N.Y. Acad. Sci. *274*, 440–450 (1976)

Fuchs, S., Nevo, D., Tarrab-Hazdai, R., Yaar, I.: Strain differences in the autoimmune response of mice to acetylcholine receptors. Nature *263*, 329–330 (1976)

Fuchs, S., Tarrab-Hazdai, R., Nevo, D., Bartfeld, D.: Experimental autoimmune myasthenia gravis: a tool for studying the pathogenesis and therapy of myasthenia gravis. In: The Biochemistry of Myasthenia Gravis and Muscular Dystrophy. *Marchbanks, R., Lunt, G.* (eds.), pp. 189–216. Academic Press 1978

Fulpius, B.W., Fontana, A., Cuenoud, S.: Central nervous system involvement in experimental autoimmune myasthenia gravis. Lancet *1977/II*, 350–351

Goldstein, G.: The thymus and neuromuscular function. Lancet *1968/II*, 119–122

Granato, D.A., Fulpius, B.W., Moody, J.F.: Experimental myasthenia in Balb/c mice immunized with rat acetylcholine receptor from denervated muscle. Proc. Natl. Acad. Sci. U.S.A. *73*, 2872–2876 (1976)

Green, D.P.L., Miledi, R., Vincent, A.: Neuromuscular transmission after immunization against acetylcholine receptors. Proc. R. Soc. Lond. [Biol.] *189*, 57–68 (1975)

Heilbronn, E.: Biochemistry of cholinergic receptors. In: Cholinergic Mechanisms. *Wasser, P.G.* (ed.) New York: Raven Press 1975, pp. 343–364

Heilbronn, E., Mattson, C., Stalberg, E., Hilton-Brown, P.: Neurophysiological signs of myasthenia in rabbits after receptor antibody development. J. Neurol. Sci. *24*, 59–64 (1975)

Heinemann, S., Bevan, S., Kullberg, R., Lindstrom, J., Rice, J.: Modulation of acetylcholine receptor by antibody against the receptor. Proc. Natl. Acad. Sci. U.S.A. *74*, 3090–3094 (1977)

Jenkins, R.B.: Treatment of myasthenia gravis with prednisone. Lancet *1972/I*, 765–767

Kalden, J.R., Williamson, W.G., Johnson, R.J., Irvine, W.J.: Studies on experimental autoimmune thymitis in guinea pigs. Clin. Exp. Immunol. *5*, 319–340 (1969)

Kao, I., Drachman, D.B.: Myasthenic immunoglubulin accelerates acetylcholine receptor degradation. Science *196*, 527–529 (1977a)

Kao, I., Drachman, D.B.: Thymic muscle cells bear acetylcholine receptors: possible relation to myasthenia gravis. Science *195*, 74–75 (1977b)

Karlin, A., Weill, C.H., McNamee, M.G., Valderrama, R.: Facets of the structures of acetylcholine receptors from Electrophorus and Torpedo. Cold Spring Harbor Symp. Quant. Biol. *40*, 203–210 (1976)

Lee, C.Y.: Chemistry and pharmacology of polypeptide toxins in snake venoms. Annu. Rev. Pharmacol. *12*, 265–286 (1972)

Lefvert, A.K., Pirskanen, K.: Acetylcholine-receptor antibodies in cerebrospinal fluid of patients with myasthenia gravis. Lancet *1977/II*, 351–352

Lennon, V.A., Lindstrom, J.M., Seybold, M.E.: Experimental autoimmune myasthenia: cellular and humoral immune responses. J. Exp. Med. *141*, 1365–1375 (1975).

Lennon, V.A., Lindstrom, J.M., Seybold, M.E.: Experimental autoimmune myasthenia gravis: cellular and humoral immune responses. Ann. N.Y. Acad. Sci. *274*, 283–299 (1976)

Lennon, V.A., Seybold, M.E., Lindstrom, J.M., Cochrane, C., Ulevitch, R.: Role of complement in the pathogenesis of experimental autoimmune myasthenia gravis. J. Exp. Med. *147*, 973–983 (1978)

Lindstrom, J.M., Einarson, B.L., Lennon, V.A., Seybold, M.E.: Pathological mechanisms in experimental autoimmune myasthenia gravis: Immunogenicity of syngeneic muscle acetylcholine receptor and quantitative extraction of receptor and antibody receptor complexes from muscles of rats with experimental autoimmune myasthenia gravis. J. Exp. Med. *144*, 726–638 (1976a)

Lindstrom, J., Einarson, B., Merlie, J.: Immunization of rats with polypeptide chains from torpedo acetylcholine receptor causes an autoimmune response to receptors in rat muscle. Proc. Natl. Acad. Sci. U.S.A. *75*, 769–773 (1978)

Lindstrom, J.M., Engel, A.G., Seybold, M.E., Lennon, V.A., Lambert, E.H.: Pathological mechanisms in experimental autoimmune myasthenia gravis. II. Passive transfer of experimental autoimmune myasthenia gravis in rats with anti-acetylcholine receptor antibodies. J. Exp. Med. *144*, 739–753 (1976b)

Lindstrom, J.M., Seybold, M.E., Lennon, V.A., Whittingham, S., Duane, D.D.: Antibody to acetylcholine receptor in myasthenia gravis: Prevalence, clinical correlates, and diagnostic value. Neurology (Minneap.) *26*, 1054–1059 (1976c)

Martinez, R.D., Tarrab-Hazdai, R., Aharonov, A., Fuchs, S.: Cytophilic antibodies in experimental autoimmune myasthenia gravis. J. Immunol. *118*, 17–20 (1977)

Matell, G., Bergstrom, K., Franksson, C., Hammarstrom, L., Lefvert, A.K., Moller, E., Von Reis, G., Smith, E.: Effects of some immunosuppressive procedures on myasthenia gravis. Ann. N.Y. Acad. Sci. *274*, 659–676 (1976)

Merlie, J.P., Sobel, A., Changeux, J.-P., Gros, F.: Synthesis of acetylcholine receptor during differentiation of cultured embryonic muscle cells. Proc. Natl. Acad. Sci. U.S.A. *72*, 4028–4032 (1975)

Nachmansohn, D.: Chemical and Molecular Basis of Nerve Activity. New York: Academic Press 1959

Nastuk, W.L., Plecia, O.J., Osserman, K.E.: Changes in serum complement activity in patients with myasthenia gravis. Proc. Soc. Exp. Biol. Med. *105*, 177–184 (1960)

Newsom Davis, J., Pinching, A.J., Vincent, A., Wilson, S.G.: Function of circulating antibody to acetylcholine receptor in myasthenia gravis investigated by plasma exchange. Neurology (Minneap.) *28*, 266–272 (1978)

Oosterhuis, H.J.G.H., Feltkamp, T.E.W., van Rossum, A.L., van den Berg-Loonen, P.M., Nijenhuis, L.E.: HL-A antigens, autoantibody production and associated diseases in thymoma patients, with an without myasthenia gravis. Ann. N.Y. Acad. Sci. *274*, 468–474 (1976)

Otterness, I.G., Chang, Y.: Comparative study of cyclophosphamide, 6-mercaptopurine, azathioprine and methotrexate: Relative effects on the humoral and the cellular immune response in the mouse. Clin. Exp. Immunol. *26*, 346–354 (1976)

Patrick, J., Lindstrom, J.: Autoimmune response to acetylcholine receptor. Science *180*, 871–872 (1973)

Patrick, J., Stallcup, W.B.: Immunological distinction between acetylcholine receptor and the α-bungarotoxin-binding component on sympathetic neurons. Proc. Natl. Acad. Sci. U.S.A. *77*, 4689–4692 (1977)

Penn, A.S., Chang, H.W., Lovelace, R.E., Niemi, W., Miranda, A.: Antibodies to acetylcholine receptors in rabbits: immunochemical and electrophysiological studies. Ann. N.Y. Acad. Sci. *274*, 354–376 (1976)

Penn, A.S., Lovelace, R.E., Lange, D.J., Toufexis, G., Brockbank, K.: Experimental myasthenia gravis in neonatally thymectomized rabbits. Neurology (Minneap.) *27*, 365 (1977)

Pinching, A.J., Peters, D.K., Newsom Davis, J.: Remission of myasthenia gravis following plasma-exchange. Lancet *1976/II*, 1373–1376

Pirskanen, R.: On the significance of HL-A and LD antigens in myasthenia gravis. Ann. N.Y. Acad. Sci. *274*, 451–460 (1976)

Prives, J., Hoffman, L.: Regulation of acetylcholine receptor biosynthesis and degradation during differentiation of muscle cells in culture. Isr. J. Med. Sci. (in press) (1978)

Prives, J., Silman, I., Amsterdam, A.: Appearance and disappearance of acetylcholine receptor during differentiation of chick skeletal muscle in vitro. Cell *7*, 543–550 (1976)

Prives, J., Hoffman, L., Amsterdam, A., Tarrab-Hazdai, R., Fuchs, S.: Ligand-induced changes in stability and distribution of acetylcholine receptors on surface membranes of muscle cells in culture. (Submitted for publication) (1978)

Raftery, M.A., Vandlen, R.L., Reed, K.L., Lee, T.: Characterization of Torpedo californica acetylcholine receptor: Its subunit composition and ligand-binding properties. Cold Spring Harbor Symp. Quant. Biol. *40*, 193–202 (1976)

Ramshaw, I.A., Bretscher, P.A., Parish, C.R.: Regulation of the immune response I. Suppres-

sion of delayed-type hypersensitivity by T cells from mice expressing humoral immunity. Eur. J. Immunol. *6*, 674–679 (1976)

Ramshaw, I.A., Bretscher, P.A., Parish, C.R.: Regulation of the immune response. II Repressor T cells in cyclophosphamide induced tolerant mice. Eur. J. Immunol. *7*, 180–185 (1977)

Richman, D.P., Patrick, J., Arnason, B.G.W.: Cellular immunity in myasthenia gravis: response to purified acetylcholine receptor and autologous thymocytes. N. Engl. J. Med. *294*, 694–698 (1976)

Schmidt-Sole, J., Tarrab-Hazdai, R., Fuchs, S.: Immunologic and physiologic activity of polyalanyl acetylcholine receptor. Isr. J. Med. Sci. *13*, 1043 (1977)

Schwartz, M., Lancet, D., Tarrab-Hazdai, R., Fuchs, S.: Effect of azathioprine on the affinity of antibodies against acetylcholine receptor: analysis with purified antibodies. (Submitted to publication) (1978 a)

Schwartz, M., Novick, D., Givol, D., Fuchs, S.: Induction of anti-idiotypic antibodies by immunization with syngeneic spleen cells educated with acetylcholine receptor. Nature *273*, 543–545 (1978 b)

Seybold, M.E., Drachman, D.B.: Gradually increasing doses of prednisone in myasthenia gravis: reducing the hazards of treatment. N. Engl. J. Med. *290*, 81–84 (1974)

Simpson, J.A.: Myasthenia gravis: a new hypothesis. Scot. Med. J. *5*, 419–436 (1960)

Sugiyama, H., Benda, P., Meunier, J.C., Changeux, J.P.: Immunological characterization of the cholinergic receptor protein from *Electrophorus electricus*. F.E.B.S. Lett. *35*, 124–128 (1973)

Tarrab-Hazdai, R., Aharonov, A., Abramsky, O., Silman, I., Fuchs, S.: Animal model for myasthenia gravis: acetylcholine receptor-induced myasthenia in rabbits, guinea pigs and monkeys. Isr. J. Med. Sci. *11*, 1390 (1975 a)

Tarrab-Hazdai, R., Aharonov, A., Abramsky, O., Yaar, I., Fuchs, S.: Passive transfer of experimental autoimmune myasthenia by lymph node cells in inbred guinea pigs. J. Exp. Med. *142*, 785–789 (1975 b)

Tarrab-Hazdai, R., Aharonov, A., Silman, I., Fuchs, S., Abramsky, O.: Experimental autoimmune myasthenia gravis induced in monkeys by purified acetylcholine receptor. Nature *256*, 128–130 (1975 c)

Tarrab-Hazdai, R., Abramsky, O., Fuchs, S.: Immunosuppression of experimental autoimmune myasthenia gravis by azathioprine. II. Evaluation of immunological mechanism. J. Immunol. *119*, 702–706 (1977 a)

Tarrab-Hazdai, R., Schwartz, M., Fuchs, S.: Immunological mechanism of the immunosuppressive treatment of experimental autoimmune myasthenia gravis. Isr. J. Med. Sci. *13*, 1043 (1977 b)

Tarrab-Hazdai, R., Geiger, B., Fuchs, S., Amsterdam, A.: Localization of acetylcholine receptor in excitable membrane from the electric organ of Torpedo: Evidence for exposure of receptor antigenic sites on both sides of the membrane. Proc. Natl. Acad. Sci. U.S.A. *75*, 2497–2501 (1978 a)

Tarrab-Hazdai, R., Yaffe, D., Fuchs, S.: Destructive effect of macrophages from acetylcholine receptor sensitized animals on muscle cell cultures. Isr. J. Med. Sci. (in press) (1978 b)

Toyka, K.V., Drachman, D.B., Griffin, D.E., Pestronk, A., Winkelstein, J.A., Fischbeck, K.H., Kao, I.: Myasthenia gravis: Study of humoral immune mechanisms by passive transfer to mice. N. Engl. J. Med. *296*, 125–131 (1977)

Warmolts, J.R., Engel, W.K.: Benefit from alternative day prednisone in myasthenia gravis. N. Engl. J. Med. *286*, 12–20 (1972)

Wekerle, H., Ketelsen, U.P.: Intrathymic pathogenesis and dual genetic control of myasthenia gravis. Lancet *1977/I*, 678–680

Wekerle, H., Paterson, B., Ketelsen, U.P., Feldman, M.: Striated muscle fibers differentiate in monolayer cultures of adult thymus reticulum. Nature *256*, 493–494 (1975)

Yaffe, D.: Rat skeletal muscle cells in Tissue Culture. In: Methods and Application. *Kruse, P.F., Patterson, M.K.* (eds.) New York: Academic Press 1973, pp. 106–114

Zurn, A.D., Fulpius, B.W.: Study of two different subpopulations of antiacetylcholine receptor antibodies in a rabbit with experimental autoimmune myasthenia gravis. Eur. J. Immunol. *8*, 529–532 (1977)

Genetics of Immunoresponsiveness to Natural Antigens in the Mouse

G. Biozzi [1], D. Mouton [1], O.A. Sant'Anna [2], H.C. Passos [3], M. Gennari [2], M.H. Reis [2], V.C.A. Ferreira [2], A.M. Heumann [1], Y. Bouthillier [1], O.M. Ibanez [2], C. Stiffel [1], and M. Siqueira [2]

[1] Service d'Immunogénétique, Institut Curie, 26, rue d'Ulm, 75231 Paris Cedex 05, France
[2] Seção de Imunologia, Instituto Biologico, P.O.B. 7119, São Paulo, Brazil. Supported in part by the Conselho Nacional de Desenvolvimento Cientifico e Tecnologico, Brazil
[3] Departamento de Microbiologia e Imunologia, Escola Paulista de Medicina, P.O.B. 7144, São Paulo, Brazil

I. Introduction

The complex and potent system of mammalian immunity has been produced by natural selection, through the progressive improvement of the less efficient mechanisms of more primitive animals. The teleonomic function of the immunity system that has directed its evolution, is the self-protection against invasion by viruses, bacteria, parasites (anti-infection immunity) and possibly by transformed malignant cells (anti-tumour immunity).

The phylogenetic evolution of the immunity system has therefore been guided by the need to adapt the host defence against diversified and increasingly sophisticated forms of aggression resulting from the host—parasite—environment equilibrium.

The most primitive type of immunity was conferred by phagocytic cells endowed with the ability to engulf and digest invading micro-organisms. This first step of immunity is essentially non-specific and lacks of memory. The second step in evolution was the elaboration of cell-mediated immunity characterized by unrefined specificity. The final step was the acquired ability to synthesize and release highly stereospecific molecules: the antibodies. The cell-mediated and humoral immunity retain memory and therefore take advantage of the immunological history characterizing each individual. Vaccination is based on this immunological memory.

In mammals these three immunity functions coexist and are performed respectively by macrophages, T lymphocytes and B lymphocytes. Each of these functions is specialized in the protection against some infections and scarcely effective or completely inefficient against others. Consequently infections may be classified into three principal types corresponding to the protective efficiency of one or the other of the three immunity functions. This is, of course, a schematic description since two immunity functions may cooperate in the defence against a given infection; nevertheless one of the three parameters is the determinant factor of the resistance.

Macrophages, T lymphocytes and B lymphocytes coexist in the peripheral lymphoid tissue and interact very closely in the induction and regulation of

the immune response either by cell surface products (receptors) or by released molecules (T factors and antibodies).

The most fundamental finding resulting from our investigations is the demonstration that these three cell types, in spite of their close functional integration in the immunoregulatory network, are distinct protagonists of immunity submitted to separate polygenic control. The resulting polymorphic regulation of immunoresponsiveness is the fundamental characteristic of the immunity system strategy ensuring the best possible multidirectional protection at the level of a genetically heterogeneous population.

In fact, this genetic arrangement provides a reasonably good resistance against all types of infection for most individuals grouped around the modal phenotypes of the three immunity parameters. As a result the bulk of the population is efficiently protected against mild endemic infections, whereas only a fraction of individuals will cope efficiently with every severe epidemic and so ensure the survival of the population (see Chapter VI).

This theory of polyvalent protective efficiency of the immunity system is based on the following fundamental findings demonstrated in Selection I: 1. The independent genetic control of humoral and cell mediated immunity (Chapter V.E); and 2. the inverse relation between genetic regulation of antibody responsiveness and macrophage activity (Chapter V.B).

Modern knowledge of the genetics of immunoresponsiveness proceeds from two different experimental approaches:

1. Production by selective breeding of high and low immune responder lines of mice that are homozygous at the loci regulating immunoresponsiveness to natural polydeterminant immunogens, as described in this review (polygenic regulation).

2. Demonstration, in inbred lines of animals, of the monogenic control operated by specific immunity response genes (Ir genes) on the response to one or a few epitopes.

Two types of specific Ir genes have been described: Ir genes controlling the immunity response that are often linked with the major histocompatibility complex (MHC), and Ir genes linked with immunoglobulin structure genes operating on the fine conformation of antibody molecules, affecting specificity, homogeneity, affinity and idiotypic antigenicity.

Specific Ir genes determine responsiveness to antigens of limited heterogeneity such as synthetic polypeptides, iso-antigens or complex antigens administered at threshold doses, where only the most potent determinant of the molecule is immunogenic (*McDevitt* and *Benacerraf,* 1969; *Sela,* 1972; *Mozes* and *Shearer,* 1972; *Benacerraf* and *Dorf,* 1974).

The principal function of specific Ir genes is regulation of interaction and differentiation of the cells participating in immunity responses (*Katz* and *Benacerraf,* 1976; *Rosenthal,* 1978).

In view of the theory formulated above, two questions may be raised: What is the selective value of specific Ir genes and what are the relations between specific Ir genes and the essentially non-specific polygenic regulation of immunoresponsiveness described in this review?

The results obtained in Selection I (Chapter III.A) demonstrate that among

the group of about ten loci controlling antibody responses, one gene is H-2 linked and another is linked with the Immunoglobulin allotype. These two genes therefore have some characteristics of the Ir genes. They account for only about 20% of the total phenotypic effect produced by the group of about ten loci regulating immunoresponsiveness to complex immunogens (Chapter III.A.3).

The selective value of specific Ir genes cannot result from the control of the immunity response to synthetic polypeptide antigens or iso-antigens in a natural environment. It could result from the control of immunity response to threshold doses of natural antigens since infections are supposed to be produced by small inoculum of pathogenic micro-organisms, mimicking threshold antigen doses.

The experimental results obtained in Selection I do not confirm this possibility. In fact, we have demonstrated that antibody response to an optimal dose of Salmonella typhimurium is of polygenic character, but at a threshold immunizing dose the response is controlled by two loci, one of which is H-2 linked (*Sant'Anna* and *Bouthillier*, unpublished results). The resistance to infection produced by a minimal inoculum of living Salmonella typhimurium is much stronger in low than in high immune responder mice (*Biozzi* et al., 1978). In fact the resistance to this type of infection is due to macrophage activity (Chapter VI.A).

In other types of infection such as by pneumococci, the antibody response to bacterial polysaccharide has a protective effect. The antibody response controlled by specific Ir genes could have a selective value in the defence against this type of infection. The results of *Howard* et al. (1972) and *Baker* et al. (1976) do not support this hypothesis, since they demonstrate that antibody response to Pneumococcus polysaccharide is a polygenic trait unlinked with either H-2 locus or Immunoglobulin allotype.

Immunoglobulin allotype linked and H-2 linked Ir genes analogous to those demonstrated in Selection I (Chapter III.A.3) may have a limited selective value as participants in the total polygenic regulation of immunity responsiveness to the antigens of bacteria and parasites studied in Chapter VI.

The selective advantage conferred by monogenic control of the immune response operated by specific Ir genes is presently not clearly understood. There is no definite evidence of MHC-linked resistance to bacterial infections, whereas the MHC may participate in anti-viral or anti-tumour immunity (*McDevitt* et al., 1974; *Zinkernagel* and *Doherty,* 1975; *Klein,* 1975; *Meruelo* et al., 1977). Ir genes could also intervene in the occurrence of auto immune diseases.

The original method used in the study of quantitative genetic regulation of immunoresponsiveness reported in this review is the production, by selective breeding, of high or low responder lines of mice to natural multideterminant immunogens administered at optimal doses. Five selective breedings are described. The first and the most extensively studied is Selection I. Selections I and II are maintained in the Department of Immunogenetics at the Institut Curie in Paris, France. Selections III and IV were carried out in the Department of Immunology of the Instituto Biologico, São Paulo, Brazil. Selection V was performed in the Department of Immunology of the Escola Paulista de Medicina, São Paulo, Brazil.

II. Methods of Selective Breeding and Genetic Analysis

The results of five selection experiments for quantitative antibody responsiveness to various antigens are described in this review article. The different antigens used were sheep erythrocytes (SE) and pigeon erythrocytes (PE) (Selection I); SE only (Selection II); flagellar (f) and somatic (s) antigens of Salmonellae (Selections III and IV respectively); bovine serum albumin (BSA) and rabbit gamma globulin (RGG) (Selection V).

The characteristics of each Selection will be described later. First, we summarize the methods of selective breeding common to the five Selections and the calculations used to analyze the results.

A. Selective Breeding

The individual antibody response was established after immunization with an optimal dose of antigen i.e. the dose inducing the highest serum antibody level in the immunization schedule chosen. The response was measured during the plateau of maximal antibody level. These experimental conditions were established in preliminary experiments in random bred mice.

The phenotypic character selected was therefore "maximal antibody response produced by an optimal immunization".

The serum antibody titre was measured by direct hemagglutination in Selections I and II; by direct flagellar agglutination in Selection III; by direct somatic agglutination in Selection IV; and by passive hemagglutination in Selection V. The technical details have been described in the corresponding references. The agglutinin titre was measured as the highest doubling serum dilution giving a positive agglutination. The results were expressed in terms of either agglutinin titre or log 2 of agglutinin titre. In previous publications relative to Selections I and II, the agglutinin titre was calculated from an 1/10 initial serum dilution while in the publications concerning Selections III, IV, and V it was calculated starting from undiluted serum. *In order to facilitate the comparison of the results obtained in the five Selections, we have expressed, in this article, all the data in terms of agglutinin titre calculated from undiluted serum.*

It should be remembered that the end point of agglutinin assay is always determined in antigen excess, therefore the two factors determining the agglutinin titre i.e. antibody concentration and antibody affinity, participate in the determination of the final score.

The initial populations used to start the selective breeding—the Foundation populations (F_0)—consisted of adult outbred albino mice produced in distinct colonies in order to obtain a large genetic variability. Only Selection II was founded on an outbred population derived from a single breeder.

The two way selective breeding for maximal or minimal antibody response was based on "individual merit" and repeated in each consecutive generation. Assortative mating of the highest responder mice produced the High line (H) and that of the lowest responder mice produced the Low line (L). In each line several pairs were culled at each generation. They were issued from different families to delay, as far as possible, the increase of consanguinity in each line.

Interline crossing and brother—sister mating were excluded during the selective breeding.

As a rule, two non cross-reacting antigens were used in each Selection. They were alternated at each generation in order to avoid the interference of maternally transmitted antibodies on the immunity responsiveness of the offspring. This method was used for the major part of Selection I and for Selections III, IV and V. To evaluate the effect of the alternate use of two non cross-reacting antigens on the response to selection, Selection II was carried out with SE only. The time interval between weaning and immunization was therefore prolonged until elimination, by natural decay, of the maternal antibody. It is evident that the alternation of two antigens speeds up the selective breeding.

B. Genetic Analysis

The results obtained in each Selection demonstrate that the "Quantitative agglutinin response" is submitted to polygenic regulation. It is determined by the cumulative effect of several independent loci occupied by alleles endowed with "good" or "bad" effects on the antibody response. The data must therefore be analyzed by the methods of quantitative genetics.

We here define the terms used in this article and mention the theoretical assumptions on which the calculations are based. For more detailed explanations see *Falconer* (1960), *Cavalli-Sforza* and *Bodmer* (1971), *Bodmer* and *Cavalli-Sforza* (1976).

In genetically heterogeneous as well as in genetically homogeneous populations of mice, the individual responses expressed as log 2 of the agglutinin titre present a normal frequency distribution. The mean titre is then close to the modal titre and the individual titres are symmetrically scattered on both sides. This is therefore an unbiased scale for genetic analysis.

The F_0 populations of the five Selections are genetically heterogeneous. Their phenotypic variance (VP) is thus due to both genetic factors: genetic variance (VG) and to all non-genetic causes of variability resulting from the environment: environmental variance (VE). The VGF_0 is due to the random distribution in the individuals of the "good" and "bad" effect alleles present in the population at an unknown frequency. The assortative mating in successive generations produces a progressive accumulation of the "good" effect alleles in the H line and of the "bad" effect alleles in the L line. The result is a progressive increase in H line, and a progressive decrease in L line, of the mean agglutinin responses, accompanied by a decrease of phenotypic variances of both lines. When the maximal interline separation is reached, which cannot be increased by continuing the selective breeding, the lines are at selection limit. They are then considered homozygous at the level of all the loci controlling the selected character. This assumption will be verified by counter-selection experiments at present under way.

The total response to selection (RT) is the interline difference at selection limit. It is due to the totalled phenotypic effects produced by all the homozygous

loci. The response to selection is also expressed as the mean response per generation (RG) obtained by dividing RT by the number of generations required to reach the selection limit.

Response to selection (R) results from the genetic pressure due to the assortative mating, measured by the selection differential (S). S is the difference between the mean value of the selected parents and that of the generation out of which they have been culled. The number of offspring per selected pair being variable, S was weighted according to litter size. The selection differential per generation (SG) is the mean S value of the generations required to reach the selection limit.

R and S can be calculated either in each line separately or in terms of interline divergence by adding the values of cumulated R or S obtained in corresponding generations of H and L lines.

The mean realized heritability (h^2) calculated by the ratio RG/SG measures the average proportion of the parental deviation which is actually inherited by the progeny. In the absence of dominance, h^2 is a measure of the additive effect of the homozygous loci occupied by "good" or "bad" effect alleles. In the foundation population therefore $VF_0 \times h^2$ is an estimation of the additive variance (VA). An estimate of the number of independent loci controlling the character (n) may be obtained by the formula

$$n = \frac{1}{8} \times \frac{RT^2}{(VF_0 \times h^2)} \tag{1}$$

The meaning and limitations of the calculation of n will be discussed later.

C. Interline Hybrids

These were produced by mating homozygous generations of H and L lines at selection limit. The following crosses were made: $(H \times L) = F_1$; $(F_1 \times F_1) = F_2$; $(F_1 \times H) = BcH$; $(F_1 \times L) = BcL$.

The same number of reciprocal crosses was always made. Since no sex linked difference was observed, the data of both sexes were pooled.

1. Global Dominance

In F_1 hybrids each locus is heterozygous. In the absence of dominance the total additive effect (a) of all the loci of homozygous parental lines is: $a = \frac{1}{2}(\bar{x}H - \bar{x}L)$, then $RT = 2a$.

The dominance of polygenic characters results from the interaction of the unknown dominance effect at the level of each heterozygous locus in F_1 hybrids. It is therefore called global dominance.

The global dominance (d) is: $d = F_1 - \frac{1}{2}(\bar{x}H + \bar{x}L)$.

The proportion of the global dominance effect in relation to the additive effect is measured by the ratio d/a. The value of d/a in the absence of overdominance is between 0 (no dominance) and $+1$ or -1 (complete dominance of high or low responsiveness respectively).

2. Variance Analysis

The environmental variance VE is the phenotypic variance of the genetically homogeneous populations: H and L lines at selection limit and their F_1 hybrids. Therefore

$$VE = \frac{VH + VL + VF_1}{3} \tag{2}$$

The phenotypic variance of the genetically heterogeneous populations F_0, F_2, BcH and BcL is due to both genetic and environmental factors.

The variance of F_2 hybrids (VF_2) is:

$$VF_2 = VA + VD + VE \tag{3}$$

where VA is the additive variance and VD is the dominance variance.

The expected contribution of all the loci to the phenotypic variance of F_2, in the absence of dominance, is the additive variance: $VAF_2 = \frac{1}{2}\sum a^2$ where a is the additive effect. VAF_2 is therefore a measure of the phenotypic difference produced by homozygous loci.

The contribution of the dominance effect, d, to the VF_2 is called dominance variance: $VDF_2 = \frac{1}{4}\sum d^2$.

The variance of each backcross is due to the difference between homozygous and heterozygous loci. VBcs (the addition of VBcH and VBcL) is then:

$$VBcs = VA + 2VD + 2VE \tag{4}$$

From Equation 3 and 4 the values of VA and VD may be directly calculated as follows:

$$VA = 2VF_2 - VBcs \tag{5}$$

$$VD = VBcs - VF_2 - VE \tag{6}$$

There is another way to calculate VA and VD using differences between means rather than variances. If we postulate that individual genes have an equivalent effect or that, if there is a variability it follows a constant pattern then

$$\frac{VD}{VA} = \frac{1}{2}\left(\frac{d}{a}\right)^2 \tag{7}$$

VD may be expressed as a function of VA in Equations 3 or 4, and VA and VD may be calculated from VF_2 or VBcs respectively.

The heritability (h^2) of the character in interline crosses (F_2 or backcrosses) is measured by the ratio of VA on their total phenotypic variance:

$$h^2 = \frac{VA}{VA + VD + VE} \tag{8}$$

If we postulate a theoretical model in which all the relevant loci are completely independent and may be occupied only by two alleles endowed with an equivalent "good" or "bad" effect, then the number of loci (n) controlling the character may be calculated as follows:

$$n = \frac{a^2}{2VA} \tag{9}$$

VA is calculated from the data of interline crosses as previously shown. The calculation of n by Equation 1 and Equation 9 should coincide if no allele were lost by genetic drift during the selective breeding and if the frequency distribution of the relevant alleles in F_0 were close to 0.5.

It should be stressed that the estimate of n is very approximate and must be considered as an order of magnitude rather than a precise figure; nevertheless it permits the comparison of the results obtained in the five Selections. In fact the estimate of n is submitted to the large experimental and sampling errors inherent to the variance calculations and is based on a simplified theoretical model in which many factors are unknown and have to be postulated. The linkages so far demonstrated afford a sound experimental support in favour of the validity of this model for the analysis of the character investigated. In Selection I where ten loci are postulated, two independent linkages were demonstrated, each one accounting for about 10% of RT (Chapter III.A).

III. Results of the Five Selective Breedings

In each Selection the character "antibody response" was submitted to polygenic regulation. The principal results obtained in the five selective breedings are described in this chapter.

A. Selection I for Agglutinin Response to Sheep and Pigeon Erythrocytes

1. Selective Breeding and Genetic Analysis

The Selection was founded on 62 random bred albino mice of both sexes obtained from several commercial breeders. The assortative mating was made by culling at least six pairs per generation in each line. The mean number of mice per generation was 50 ± 15 in H line and 46 ± 15 in L line.

Fertility and fecundity were not appreciably affected by the selective breeding.

The magnitude and the kinetics of agglutinin response were both progressively modified under the effect of selection. Thus, the initial exponential rise of serum agglutinins observed until the 5th day post-immunization in the two lines was followed by a rapid fall in L responders, whilst until the 14th day, an additional rise was observed in H responders (see Fig. 5) (*Biozzi* et al., 1971, 1974).

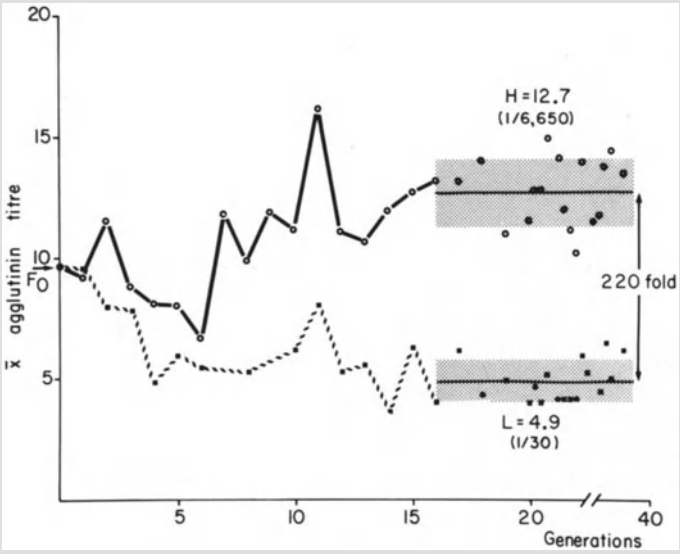

Fig. 1. Selection I. $\bar{x}$ anti SE or PE agglutinin titres (log 2) 14th day post i.v. immunization in successive generations of H and L lines. F_0–F_{16}: Divergence of H and L lines. F_{16}–F_{38}: Total range of interline separation in homozygous generations $\pm$ standard deviation

The largest phenotypic difference between H and L lines was found at the 14th day post primary immunization, therefore the results of the selective breeding were calculated according to the 14th day agglutinin titre (*Feingold* et al., 1976). The mean and variance calculations of each generation were made from individual data of both sexes since no constant sex effect was noticed in the generations. A slight female superiority of about 0.5 proved significant only when large populations (>100 mice) were compared.

The results of the selective breeding are represented in Fig. 1.

The agglutinin response of the F_0 population immunized i.v. with 1×10^8 SE was 9.7 ± 1.6. The offspring were weaned when 30 days old and immunized 10 days after weaning. This was repeated for the first six generations. Although the selective breeding produced a significant interline separation, the responsiveness of H line decreased progressively. This depression was due to the effect of a maternally transmitted antibody rather than to an inbreeding depression. In fact a strong response of H line and a large interline difference was observed in groups of F_6 mice immunized with pigeon erythrocytes (PE) that are antigenically unrelated with SE. The 7th generation was therefore immunized i.v. with the optimal dose of 10^8 PE. Afterwards the two immunogens were alternated at each generation in order to avoid the specific effect of passively transmitted maternal antibodies. This antigen alternation speeds up the selective breeding since mice may be immunized shortly after weaning (15 days). After the 13th generation the dose of SE was raised to 5×10^8.

The two lines diverged progressively during the selective breeding until the 16th generation when the maximal interline separation was obtained (selection limit). Afterwards the interline difference remained roughly constant in spite

of the continuation of the selective breeding until F_{38}. These findings may be interpreted as follows: by F_{16} the "good effect" alleles were accumulated in the H line and the "bad effect" alleles in the L line. Both lines could thus be considered as homozygous for the loci controlling the character investigated.

This group of loci produced a very large phenotypic effect since there is a 220-fold difference in agglutinin titre between H and L lines.

The response to selective breeding was asymmetrical. In relation to the F_0 population the immune responsiveness of L line was decreased 28-fold while that of H line was increased only 8-fold in terms of agglutinin titre.

The following considerations on the phenotypic variance only concerns the response to SE since the F_0 population and the interline hybrids analyzed later (Table 1), were immunized with this antigen.

The phenotypic variance of the F_0 population was 2.56. Since this is a genetically heterogeneous population, its variance is due to both genetic and environmental factors: VG and VE respectively. The variance of the successive generations decreased progressively during the selective breeding as their genetic homogeneity increased. It remained fairly constant in the homozygous generations (F_{16}–F_{38}) in which it was produced only by environmental effects (VE). The mean VE of the F_{16}–F_{38} generations immunized with SE was 0.74 in H line and 1.30 in L line. The mean in the two lines was 1.02. From this value it may be calculated that 60% of the VF_0 is attributable to genetic factors ($VGF_0 = 1.54$) and 40% produced by environmental effects.

Environmental factors are responsible for the erratic fluctuations affecting H and L lines alike. Their impact may be reduced if the response to selection is expressed in terms of interline difference, as shown in Fig. 2.

The response to selection R is the difference between the mean agglutinin titre of H and L lines of the same generation. It was cumulated at each generation (cumulated R). The selection differential S is the sum of the S values calculated separately in H and L lines at each generation (cumulated S). The mean values of R and S per generation, RG and SG respectively, were calculated by a least square linear regression from the F_0–F_{16} generations. SG measures the mean value of the selective pressure which produced the mean response to selection, RG.

The results represented in Fig. 2 show that H and L lines diverged progressively during 16 generations of selective breeding. The selection limit was reached in F_{16} where the maximal interline separation was obtained ($RT = 7.8$). From F_{16} onwards the RT value remained constant in spite of the continuation of the selective breeding that produced a steady increase of cumulated S until F_{38}. The dissociation of R from S after selection limit demonstrates that the generations between F_{16} and F_{38} are genetically homogeneous. Their phenotypic variance, entirely due to environmental factors, has no effect on their progeny.

The mean heritability (h^2) realized during the 16 generations of operative genetic selection calculated by a least square linear regression of R/S, was 0.20 ± 0.08. This value represents the mean h^2 of H and L lines since it was calculated from the interline divergence. Because of the asymmetrical response to selection previously mentioned, each line has a distinct h^2. In relation to

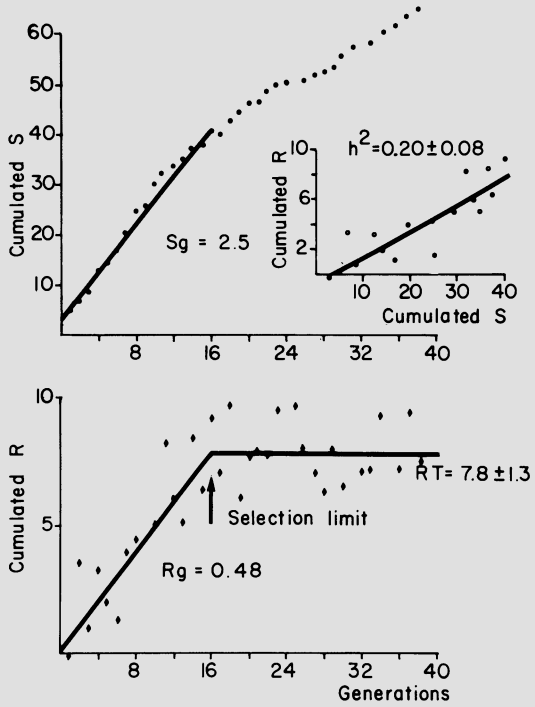

Fig. 2. Genetic analysis of Selection I — cumulated selection differential (S) log 2, in H and L lines — cumulated response to selection (R) log 2, in H and L lines. Calculation of realized heritability (h^2): plot of cumulated R on cumulated S for generations up to selection limit

the F_0 population the RT was 3.0 in H line and 4.8 in L line. The cumulated S in F_{15} was 20.2 and 19.7 in H and L lines respectively. Consequently h^2 was 0.15 in H line and 0.24 in L line. The difference in the h^2 values of H and L lines is mostly due to the incomplete dominance of high responsiveness (Table 1), which limits the response to selection in the direction of the dominance effect.

According to Equation 1 the number of independent loci may be calculated from RT, h^2 and VF_0. The result of this calculation indicates that a group of about 15 independent loci regulates the agglutinin responsiveness to SE. This estimate will be compared with the results obtained in interline hybrids (Table 1).

2. Interline Crosses

The agglutinin response to SE was measured in interline hybrids: F_1, F_2, and in the 2 backcrosses, BcH and BcL.

The following genetic analysis is based on the probable assumption that H and L lines at selection limit are homozygous at the level of all the loci controlling the character investigated.

Table 1. Mean agglutinin titres and variances in homozygous generations of H and L lines and in interline hybrids of Selection I (14th day post-immunization with the optimal dose of 5×10^8 SE)

Line	Number of mice	Log 2 agglutinin titre	
		Mean $\bar{x}$	Variance V
H line F_{16}–F_{36}	472	12.50	0.74
L line F_{16}–F_{36}	497	4.67	1.30
F_1 (H × L)	211	9.63	1.62
F_2 ($F_1 \times F_1$)	363	8.62	1.97
BcH ($F_1 \times$ H)	166	11.36	0.99
BcL ($F_1 \times$ L)	168	7.59	2.30

a = 3.915 d = 1.045 d/a = 0.27

The results concerning H and L lines were established as the mean values of all the homozygous generations immunized with SE. These data and the results obtained in interline crosses are reported in Table 1 (see also *Biozzi* et al., 1979).

a) Evaluation of the Global Dominance

The data in Table 1 show that the mean response of F_1 hybrids was closer to that of H than to that of L line. Hence there is an incomplete dominance effect of high over low responsiveness (d/a = 0.27). The dominance effect is 27% of the additive deviation, a. In F_1 where each locus is supposed to be heterozygous, the frequency distribution of the alleles in the F_1 population is 0.5. The mean of the F_0 population ($\bar{x}F_0 = 9.7$) was very close to that of F_1, therefore the frequency distribution of the relevant alleles in the F_0 population should also be close to 0.5. As mentioned before (paragraph 1) it is very probable that the asymmetrical response to selection is essentially due to the dominance effect. This conclusion eliminates the other possible causes of the asymmetrical response namely: uneven distribution of relevant alleles in the F_0 population and intervention of a different number of loci in H and L lines.

The mean responses of the other interline crosses F_2, BcH and BcL are also affected by the incomplete dominance of high responsiveness demonstrated in F_1. The mean ratio of d/a measured in F_1, F_2, BcH and BcL is 0.30 ± 0.18.

The environmental variance (VE) measured as the mean of the three genetically homogeneous populations (H, L and F_1) is 1.22 (Equation 2). The partition

Table 2. Comparison of the different estimates of variance components, heritability and number of loci in Selection I

Environmental variance VE Equation 2	Method of calculation of VA and VD	Partition of genetic variance VG		Heritability h^2 Equation 8	Number of loci n Equation 9
		Additive variance VA	Dominance variance VD		
1.22	Ⓐ From VF_2 and VB_{CS} Equations 5 and 6	0.65	0.10	0.33	11.7
	Ⓑ In VF_2 Equations 3 and 7	0.72	0.026	0.36	10.6
	Ⓒ In VBcs Equations 4 and 7	0.79	0.029	0.39	9.7
	Ⓓ In F_0 Equation 1	0.51	0.83	0.20*	14.8

* Mean value of h^2 realized during the selective breeding (Fig. 1)

between the genetic and the environmental origin of the variance is, in F_2: VG=38% and VE=62%; in BcL: VG=47% and VE=53%. Because of the dominance of high responsiveness the variance of BcH is within the range of VE values. These results underline the large impact of environmental factors in the phenotypic variability of interline segregants.

b) Evaluation of the Components of the Phenotypic Variance, Heritability and Number of Relevant Loci

The results of the variance analysis of the agglutinin response in interline hybrids are shown in Table 2. This type of analysis is subject to large sampling and experimental errors. Nevertheless the results are reasonably consistent.

The evaluation of VA and VD made according to the three methods of calculation, A, B and C, indicated in Table 2, gives concordant results. The difference in VA figures is not significant since the sampling error of VA calculated by method A is 0.13. The genetic variance of F_2 and backcrosses is almost entirely due to the additive effect; the contribution of the dominance variance (VD) is 15% according to calculation A and 4% according to calculations B and C. Because of the constancy of the VA estimate, the evaluation of h^2 in interline crosses by the three methods of calculation, A, B and C, gives similar results. The results obtained by method D differ slightly but are consistent with those mentioned above. In fact the VA established by calculation D concerns the F_0 population and the h^2 refers to the h^2 realized during the selective breeding (Fig. 2). The mean value of VA and h^2 obtained by the four estimations shown in Table 2 is: $VA=0.67\pm0.11$, $h^2=0.28\pm0.07$. It is remarkable that the estimate of n calculated from the data obtained during the selective breeding (Equation 1) coincides with that made in interline hybrids (Equation 9).

The final conclusion is that the quantitative agglutinin response to SE is regulated by a group of about ten independent loci. This estimate is confirmed by the results of the two distinct linkages demonstrated in interline segregants.

3. Demonstration of Two Independent Linkages

Two independent linkages have been demonstrated so far for two loci among the group of ten which regulate the immune response in H and L mice.

a) Immunoglobulin Allotype Linked Locus

It has been recognized that H and L mice differ in the Ig heavy chain structure genes (*Biozzi* et al., 1970). The study of agglutinin response to SE in interline F_2 hybrids and in backcrosses demonstrated a positive correlation between the Ig allotype distribution and the agglutinin level (*Lieberman* et al., 1972). The mean agglutinin titre in F_2 hybrids homozygous for the H line Ig allotype was 9.8 whereas it was 8.8 in F_2 hybrids homozygous for the L line Ig allotype ($p<0.01$). This difference compared with the RT value (7.8) means that the quantitative contribution of the allotype linked locus is 13% of the total phenotypic difference between homozygous H and L lines.

b) H-2 Linked Locus

Experiments of skin graft exchanged between H and L lines and serum lymphocytotoxicity tests demonstrated that the two lines differ at the major histocompatibility locus H-2 (*Liacopoulos-Briot* et al., 1972). The distribution of H and L line H-2 phenotypes in F_2 hybrids and backcrosses was established by a lymphocyte cytotoxicity test in presence of C′ using specific allo antisera.

A positive correlation was found in F_2 hybrids and backcrosses, between the H-2 phenotype and the SE agglutinin response. In F_2 hybrids homozygous at the H-2 locus of H line the SE agglutinin titre was 10.7 and it was 9.3 in F_2 mice homozygous at the L line H-2 locus ($p<0.001$). The phenotypic effect produced by the H-2 linked locus was 1.4.

This difference is 18% of that separating H and L lines homozygous at the level of all the ten loci regulating SE responsiveness ($RT=7.8$) (*Stiffel* et al., 1974). In other experiments described in paragraph 4 the contribution of the H-2 linked locus was estimated as 10% of the RT value.

As mentioned above, the quantitative effect of these two identified loci confirms the estimate of the total number of relevant loci since the model used to calculate n postulates that each locus has an equivalent effect of 10% of RT value.

4. Genetic Control of Responsiveness to Threshold Doses of Sheep Erythrocytes

The results described so far concern the response to an optimal dose of SE, i.e., the character used to carry out the selective breeding.

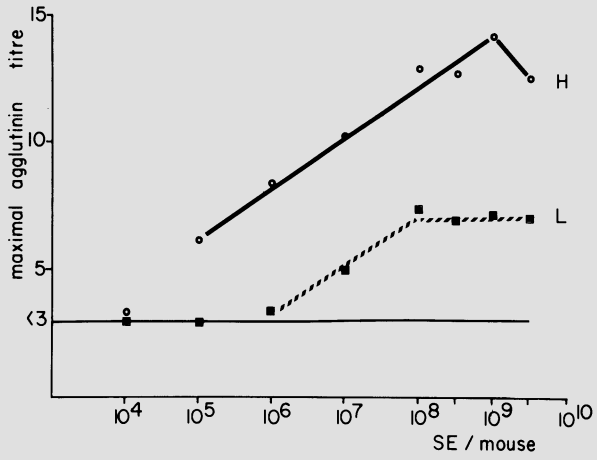

Fig. 3. Dose-response relationship in H and L lines of Selection I: peak agglutinin titres (log 2) in groups of 5–10 mice from each line, immunized i.v. with increasing doses of SE (titres < 3 = background titre)

In other experiments the antibody response was measured in groups of H and L mice immunized with a large range of SE doses, from sub-immunogenic to supra-maximal. The peak agglutinin titres in both lines for each antigen dose are shown in Fig. 3.

In the range of dose-response relationship, the interline difference was constant and independent from the antigen dose (*Biozzi* et al., 1972a). The sensitivity to immunogenic stimulation was very different in the two lines. The threshold dose of antigen required to induce a detectable response was about 100-fold lower in H than in L mice. For the dose of 10^6 SE, H and L mice could be classified as "responders" and "non-responders" respectively, since the titre of natural SE agglutinin is $\leqq 3$.

The inheritance of the threshold character "responsiveness to 10^6 SE" investigated in interline hybrids, F_1, F_2 and both backcrosses is presented in Table 3.

It is evident that the genetic regulation of responsiveness to 10^6 SE is quite different from that described for the optimal dose of SE (Tables 1 and 2). The two fundamental differences concern the dominance and the number of loci involved. At the optimal immunizing dose: 5×10^8 SE, there was an incomplete dominance effect of high response in F_1 hybrids: $d/a = 0.27$ (Table 1). This effect was reversed by decreasing the dose of SE: for the dose of 10^6 SE, $d/a = -0.53$ and for 10^5 SE a complete dominance of low responsiveness was observed.

The number of relevant loci, n, can be evaluated either by the variance analysis as reported in Table 3, or by comparing the distribution of parental phenotypes in segregant interline hybrids with that expected according to Mendelian inheritance (*Stiffel* et al., 1974). Both methods give concordant results which demonstrate that two loci intervene in the control of the antibody response to 10^6 SE.

Table 3. Mean agglutinin titres and variances in H, L and interline hybrids of Selection I (10th day, post-immunization with the threshold dose of 10^6 SE). Resulting genetic parameters

Line	Number of mice	Log 2 agglutinin titre	
		Mean $\bar{x}$	Variance V
H line	40	8.4	2.94
L line	41	3.56	0.29
F_1 ($H \times L$)	31	4.7	1.60
F_2 ($F_1 \times F_1$)	120	5.9	3.77
BcH ($F_1 \times H$)	42	6.60	4.95
BcL ($F_1 \times L$)	43	3.53	0.85

$a = 2.42$ $d = -1.28$ $d/a = -0.53$
$VA = 1.6$ (Equation 5), $n = 1.8$ (Equation 9)

The antibody response to the optimal dose of 5×10^8 SE is controlled by about ten loci (Table 2), one of which is H-2 linked whereas only two loci intervene in the control of the response to the threshold dose of 10^6 SE. It was important to investigate whether one of the 2 loci was H-2 linked. In order to study the quantitative effect of H and L H-2 phenotypes in large groups of mice, the following experiment was designed. F_2 mice homozygous for either H or L phenotypes were mated. They produced two populations referred to as F_3 H/H and F_3 L/L respectively. These mice differ only at the level of the H-2 locus, while the background genes as well as the other genes controlling the immunity regulation are distributed at random as in an F_2 population. Thus the mean difference between the two groups of F_3 H/H and F_3 L/L is an exact measure of the H-2 linked locus effect. These two populations were immunized with either the optimal (5×10^8) or the threshold (10^6) dose of SE. The results are shown in Fig. 4 as individual maximal agglutinin titres.

The difference between the mean responses to 5×10^8 SE in F_3 H/H and F_3 L/L is significant ($p < 0.02$); it is equal to 10% of the interline difference between the two homozygous lines ($RT = 7.8$, Fig. 2). This estimation is consistent with the previously mentioned data obtained in F_2 hybrids differing at the level of the H-2 locus.

A greater difference in agglutinin response was observed between F_3 H/H and F_3 L/L immunized with 10^6 SE. It was evident that almost all the mice homozygous for the H-2 phenotype of H line were "responders" to this antigen dose, as H mice are. On the contrary, in F_3 L/L mice, an important percentage, but not all, were "non-responders", as L mice are. This finding is explained by

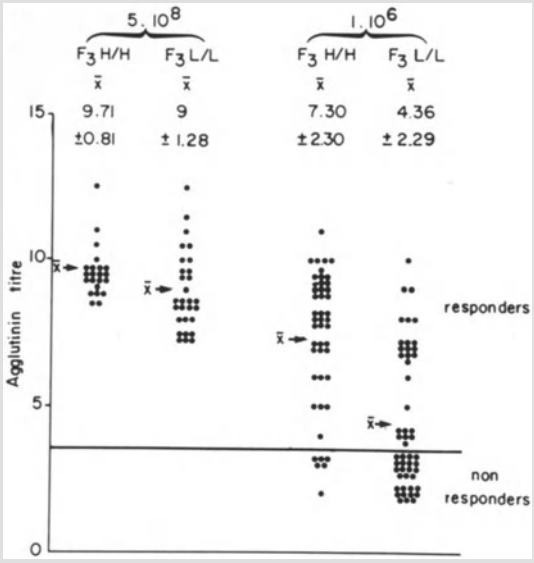

Fig. 4. Individual maximal agglutinin titres (log 2) in F_3 H/H and F_3 L/L mice (having the H-2 phenotype of H and L mice respectively on a F_2 background) immunized i.v. with 5×10^8 or 10^6 SE (mice with titres $\leqq 3$ are classified "non responders" as L mice)

the intervention of the second locus participating in the regulation of responsiveness to the threshold dose of SE. 73% of the total number of F_3 H/H and F_3 L/L mice gave the response expected according to the parental H-2 phenotype. The difference between the mean agglutinin titres in F_3 H/H and F_3 L/L compared with that observed between H and L lines, for the same antigen dose, demonstrated that 61% of the interline difference is due to the H-2 linked locus (*Mouton* et al., to be published).

The quantitative effect of the H-2 linked locus is 10% of the interline difference for the optimal dose of SE and 61% of the interline difference for the threshold dose of SE. Since the regulation is operated by ten loci in the first instance and by two loci in the second, the quantitative contribution of the H-2 linked locus fits with the calculations of the number of loci made by the variance analysis (Tables 2 and 3).

These results deserve the following concluding remarks. The complexity of the genetic regulation of antibody response to SE decreases with the dose of antigen administered. Ten loci operate at the optimal immunizing dose and only two loci at the threshold dose. For both doses an H-2 linked locus is involved. The H-2 linked locus operating at the threshold dose of SE has some characteristics of a specific H-2 linked Ir gene discriminating responders from non-responders to threshold doses of native antigens (*Vaz* et al., 1970, 1971; *Benacerraf* and *McDevitt*, 1972; *Benacerraf*, 1973). Two important differences between our model and the monogenic control operated by Ir genes should be stressed: the intervention of a second locus for the responsiveness to the threshold dose of SE and the opposite dominance effect. In the H-2

linked specific Ir model the high response is dominant in F_1 hybrids whereas in our model the low response is dominant. Another similar example concerning the responsiveness to small doses of hen egg albumin in H and L lines is reported in Chapter IV.A.

5. Kinetics of 19S and 7S Agglutinin Production During Primary and Secondary Responses

Selective breeding was carried out for the agglutinin titre during the primary response. The results reported in Fig. 5 demonstrate that both primary and secondary responses to SE are modified in H and L lines at selection limit (*Biozzi* et al., 1974).

The kinetics of the total agglutinin response after primary immunization was very different in H and in L lines (Fig. 5A). After an initial exponential rise for 4–5 days in both lines the agglutinin level decreased rapidly in L line, whereas it increased and persisted at very high levels in H line. After the second challenge both lines presented a secondary response though the interline difference was smaller than after primary immunization.

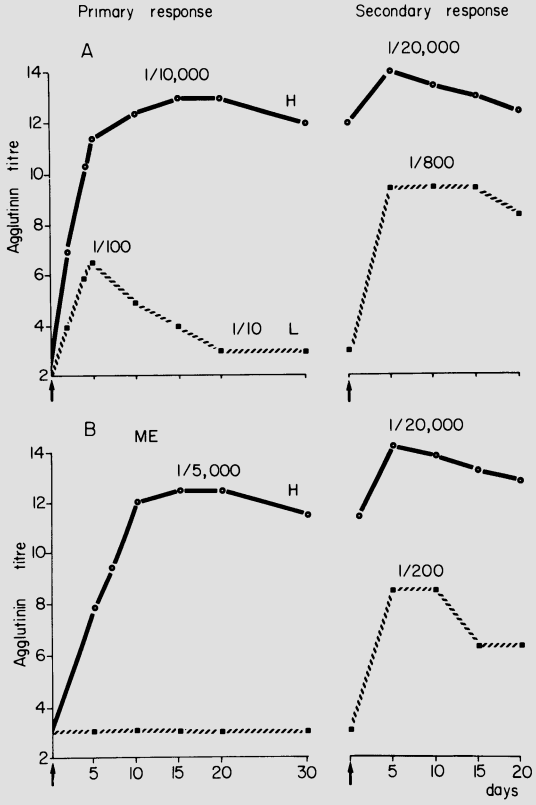

Fig. 5. Kinetics of primary and secondary responses to i.v. immunization with 5×10^8 SE in H and L lines of Selection I. $\bar{x}$ titres (log 2) of total (A) and ME resistant (B) agglutinin in groups of 10–15 mice from each line

These results indicate that both primary and secondary responses are submitted, at least partially, to the same genetic control.

Figure 5 B represents the kinetics of primary and secondary agglutinin responses established in samples of immuneserum treated with mercaptoethanol (ME) in order to destroy the 19 S agglutinins.

During the primary response H mice produced both 19 S and 7 S agglutinins with a quantitative preponderance of the latter. L mice on the contrary only produced 19 S agglutinins.

During the secondary response only 7 S antibodies were synthesized in H line whilst both antibody classes were produced in L line.

These findings indicate that the genes regulating immunoresponsiveness operate on the synthesis of the two major classes of antibody: 19 S and 7 S.

The effect on the other classes and sub-classes of antibody are described in Chapter V.C.

B. Selection II for Agglutinin Response to Sheep Erythrocytes

1. Selective Breeding and Genetic Analysis

In this Selection all the generations were immunized with the same antigen: SE. The mice were weaned 30 days after birth. The period between weaning and immunization was extended to 60–70 days in order to eliminate the maternal antibodies. The F_0 population consisted of 50 random bred albino mice of both sexes obtained from a single commercial breeder. At least seven pairs per generation were selected in each line. The mean number of mice per generation was 56 ± 13 in H line and 58 ± 14 in L line.

The immunization was given intravenously. The SE dose was 10^8 until the sixth generation and 5×10^8 afterwards.

The kinetics of agglutinin response measured in H and L lines at selection limit was very similar to that of Selection I reported in Fig. 5. As for Selection I, the greatest difference between H and L responders was found 14 days after primary immunization; therefore the 14th day agglutinin titer was considered as the phenotypic measure for the agglutinin responsiveness. As for Selection I a small superiority in the responses of female mice was noticed. The mean of this sex effect calculated in the 22nd generation of H and L lines was 0.6. The means and variances of each generation were calculated from individual data of both sexes (*Feingold* et al., 1976).

The results of the selective breeding are shown in Fig. 6.

The response of the F_0 population was 10.1 ± 1.56. It was almost identical to that of the F_0 population of Selection I, which suggests a similar genetic constitution of the two F_0 populations.

The H and L lines diverged progressively during the first 13 generations. Afterwards, the interline difference remained roughly constant in spite of the continuation of the selective breeding until F_{22}. Selection limit was reached in F_{13}.

The mean response of the generations considered as homozygous for the genes controlling the responsiveness (F_{14}–F_{22}) was 11.6 in H line and 4.9 in

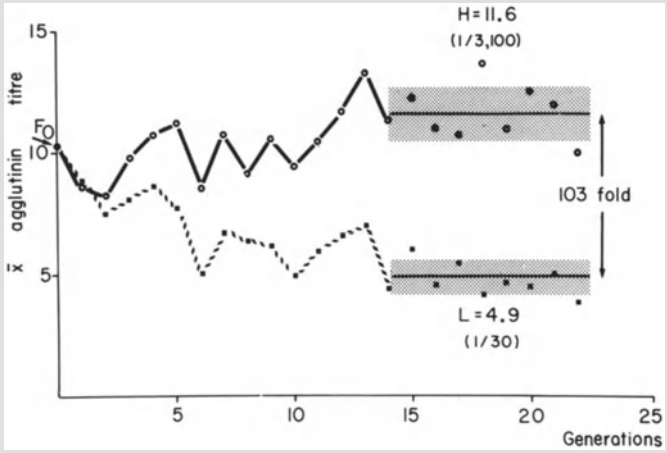

Fig. 6. Selection II. $\bar{x}$ anti SE agglutinin titres (log 2) 14th day post i.v. immunization in successive generations of H and L lines. F_0–F_{14}: Divergence of H and L lines. F_{14}–F_{22}: Total range of interline separation in homozygous generations $\pm$ standard deviation

L line. The maximal interline separation was 103-fold in terms of agglutinin titre.

In Selection II also there was an asymmetrical effect of the selective breeding which produced a greater downwards than upwards response to selection in relation to the level of the F_0 population. In fact the responsiveness of L line was decreased 36-fold whereas that of H line was increased only 3-fold.

The environmental variance, VE, calculated as the mean of the homozygous generations (F_{14}–F_{22}) of the two lines was 1.24; the phenotypic variance of the F_0 population VF_0 was 2.43. Therefore 49% of the VF_0 is due to genetic factors ($VGF_0 = 1.19$) and 51% to environmental effects.

The response of F_1 interline hybrids (10.06) (Table 4) was identical to that of the F_0 population, therefore the asymmetry is due to the dominant effect of high responsiveness.

The cumulated response to Selection (R), measured as interline difference and the cumulated Selection differential (S), of the two lines are shown in Fig. 7.

A mean response to selection of 0.43 was obtained by exerting a mean genetic pressure of 2.24 per generation. Selection limit was reached in F_{13}. The mean value of RT calculated in the 13 generations at selection limit was 6.7 ± 1.3.

The heritability realized during the 13 generations of interline separation was 0.21 ± 0.05. It was determined by a least square linear regression of R/S. This value represents the mean h^2 of H and L lines calculated from the interline divergence.

The value of h^2 in each line was influenced by the dominance effect responsible for the asymmetrical response to selection. In relation to the level of the F_0 population (10.1) the RT was 1.4 in H line and 5.3 in L line. The cumulated S was 12.0 and 19.6 respectively. Then h^2 is 0.12 in H line and 0.27 in L line.

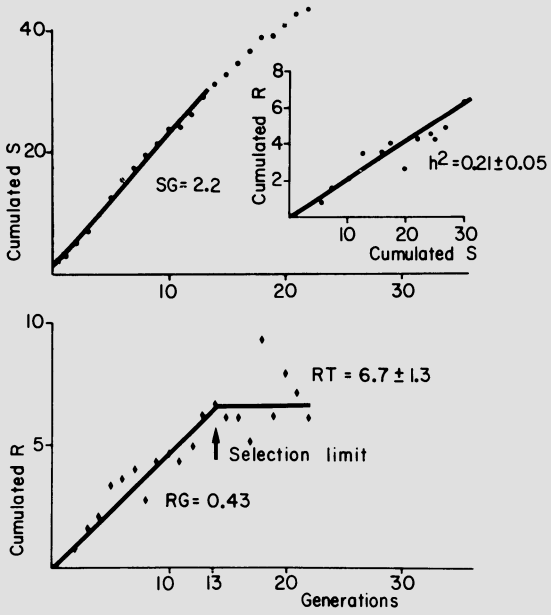

Fig. 7. Genetic analysis of Selection II — cumulated selection differential (S) log 2, in H and L lines — cumulated response to selection (R) log 2, in H and L lines. Calculation of realized heritability (h^2): plot of cumulated R on cumulated S for generations up to selection limit

2. Interline Crosses

The agglutinin responses to SE measured in interline hybrids: F_1, F_2 and in the two backcrosses: BcH and BcL are reported in Table 4 (see also *Biozzi* et al., 1979).

a) Evaluation of the Global Dominance

The dominance of high over low responsiveness measured by the ratio d/a in F_1 hybrids was 0.54. The mean value of dominance measured in F_1, F_2, BcH and BcL was 0.51 ± 0.23. This dominance effect explains why the variance of BcL is larger than that of BcH.

The environmental variance, VE, measured as the mean of the genetically homogeneous population of H, L and F_1 hybrids was 1.31 (Equation 2).

The partition between the genetic and environmental origin of the variance was: in F_2, VG=69% and VE=31%; in BcL, VG=78% and VE=22%. The genetic component of the variability in interline segregant hybrids in therefore larger in Selection II than in Selection I.

The VE values of homozygous generations at selection limit were similar in Selections I and II. The dominance effect of high responsiveness is about 2-fold larger in Selection II than in Selection I. This explains the larger asymmetry in the response to selection observed in Selection II.

Table 4. Mean agglutinin titres and variances in homozygous generations of H and L lines and in interline hybrids of Selection II (14th day post-immunization with the optimal dose of 5×10^8 SE)

Line	Number of mice	Log 2 agglutinin titre	
		Mean $\bar{x}$	Variance V
H line F_{14}–F_{22}	466	11.60	1.01
L line F_{14}–F_{22}	455	4.90	1.47
F_1 (H × L)	88	10.06	1.46
F_2 ($F_1 \times F_1$)	171	9.54	4.22
BcH ($F_1 \times$ H)	88	10.27	1.76
BcL ($F_1 \times$ L)	146	7.47	5.89
a = 3.35	d = 1.81	d/a = 0.54	

Table 5. Comparison of the different estimates of variance components, heritability and number of loci in Selection II

Environmental variance VE Equation 2	Method of calculation of VA and VD	Partition of genetic variance VG		Heritability h^2 Equation 8	Number of loci n Equation 9
		Additive variance VA	Dominance variance VD		
1.31	Ⓐ From VF_2 and VBcs Equations 5 and 6	0.95	1.94	0.23	5.9
	Ⓑ In VF_2 Equations 3 and 7	2.50	0.38	0.60	2.3
	Ⓒ In VBcs Equations 4 and 7	3.70	0.56	0.66	1.5
	Ⓓ In F_0 Equation 1	0.51	0.61	0.21*	10.9

* Mean value of h^2 realized during the selective breeding (Fig. 6)

b) Evaluation of the Components of the Phenotypic Variance, Heritability and Number of Relevant Loci

The results of the variance analysis of H and L lines and their crosses F_1, F_2, BcH and BcL are reported in Table 5.

The three methods of calculation, A, B and C, give less satisfactory results than those obtained in Selection I (Table 2). Actually, the VA values resulting from calculations B and C are larger than that obtained from calculation A, and give high h^2 values and small numbers of loci. These results greatly contrast with those obtained during the selective breeding. In fact, if the h^2 were 0.60, the selection limit (RT=6.7) should have been reached by the 5th generation instead of the 13th as actually observed (Fig. 7).

On the contrary, the h^2 realized during the selective breeding ($h^2 = 0.21 \pm 0.05$) is a sound experimental result corresponding to the h^2 evaluated in interline hybrids by calculation A.

The results of calculations A and D are based on independent experimental data, while those of calculations B and C rest upon the assumption implicit in Equation 7. A possible explanation for this discrepancy is given in *Biozzi* et al. (1979).

At present the most probable hypothesis is that the number of independent loci regulating agglutinin responsiveness in Selection II is between six and ten.

The study of linkages with the Ig allotype and with the H-2 locus, and their quantitative contribution to the total interline separation is in progress. It will give experimental evidence in favour of or against the numer of loci hypothesized.

C. Selection for Agglutinin Response to Salmonella Antigen

Two antigenically distinct Salmonellae were alternated at each generation: Salmonella typhimurium (Salm. tm.) and Salmonella oranienburg (Salm. or.). Both Salmonellae contained flagellar (f) and somatic (s) antigens.

Preliminary experiments demonstrated that Salm. tm. and Salm. or. had no cross-reacting antigens in mice and that f and s agglutinin responses were completely independent from each other.

Selective breeding was carried out for the character "peak agglutinin titre" in response to a secondary optimal immunization. The optimal dose was 3.3×10^8 for Salm. tm. and 1×10^9 for Salm. or. Two intraperitoneal injections of formalin-killed bacteria were given 8 days apart. The maximal agglutinin response to both f and s antigens was reached 10 days after the second immunization and measured separately according to the techniques described (*Siqueira* et al., 1976).

From a common F_0 population, two distinct two-way selections were made, one for responsiveness to f antigen (Selection III) and the other for responsiveness to s antigen (Selection IV). Four lines of mice were therefore produced: an H and an L responder line to f antigen, and an H and an L responder line to s antigen. In each selection the agglutinin response to both f and s antigens was measured, one being the "selection antigen" and the other the "associated antigen". Since f and s are completely independent antigens the comparison of the response to the selection antigen and to the associated antigen will give useful information on the specificity of the selective breeding (Chapter IV.C).

The F_0 population common to Selections III and IV was constituted by 75 outbred albino mice obtained from four independent breeding colonies.

1. Selection III for Agglutinin Response to f Antigen of Salmonella

a) Selective Breeding and Genetic Analysis

The F_0 population was immunized with Salm. tm. Then the two Samonellae were alternated at each generation. The individual titres (log 2) of f agglutinins presented a normal distribution in F_0: $\bar{x} = 10.3 \pm 1.21$. The f agglutinin response to Salm. or. was measured in 80 mice of the same origin as the F_0 population. The result was $\bar{x} = 10.9 \pm 1.33$. The equivalence of agglutinin response to Salm. tm. and Salm. or. was confirmed in the successive generations. The data obtained during the selective breeding with both Salmonellae may therefore be cumulated.

A small mean superiority of female responses of about 0.6 log 2 was noticed. The calculations were made from individual data of male and female mice from each generation, both sexes being nearly equally represented.

The mean number of mice per generation was 64 ± 13 in H line and 60 ± 14 in L line. In each line a mean of eight reproductive pairs was selected at each generation.

The results of interline separation produced by 19 consecutive generations of selective breeding are represented in Fig. 8. The phenotypic variance is similar

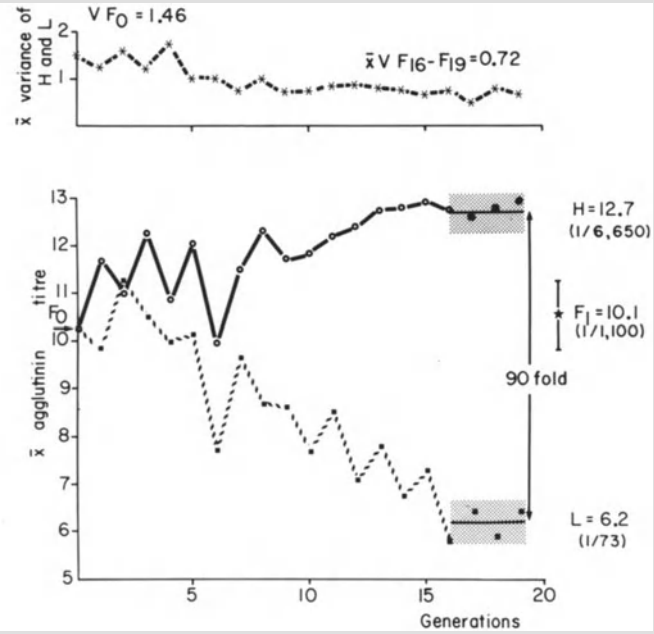

Fig. 8. Selection III. $\bar{x}$ anti f agglutinin titres (log 2) 10 days post 2 i.p. injections (8 days apart) of 3.3×10^8 Salm. tm. or 10^9 Salm. or., in successive generations of H and L lines. F_0–F_{16}: Divergence of H and L lines. F_{16}–F_{19}: Total range of interline separation in homozygous generations $\pm$ standard deviation. Upper part: $\bar{x}$ of H and L variances in corresponding generations

in corresponding generations of each line, therefore their mean value is represented in the upper part of Fig. 8.

H and L responder lines diverged progressively from each other during 16 generations, then in spite of the continuation of the selective breeding up to F_{19} the interline separation remained roughly constant. Selection limit was thus reached in F_{16} (Fig. 9) (*Siqueira* et al., 1976).

The four generations at selection limit, F_{16}–F_{19}, were considered homozygous at the level of all the relevant loci. Their mean response was 12.7 in H line and 6.2 in L line; this makes an interline difference of 90-fold in terms of agglutinin titres. F_1 interline hybrids were produced from F_{19} H and L mice. The f agglutinin response of 86 F_1 was 10.1 ± 0.81. The mean responses of the F_0 population and F_1 hybrids were identical, so in both populations the frequency distribution of the relevant alleles is supposed to be similar and close to the intermediate value of 0.5. The mean agglutinin titre of F_1 was closer to that of H than L homozygous generations, which indicates an incomplete dominance of high over low responsiveness.

The global dominance effect in F_1 hybrids is 20% of the additive effect: $d/a = 0.2$.

The response to selection was asymmetrical. In relation to the level of the F_0 population, agglutinin responsiveness was increased by 5-fold in H line and decreased by 18-fold in L line. Because of the reasons previously discussed, this asymmetrical effect is probably due to the incomplete dominance of the high response.

The mean phenotypic variance produced by environmental factors (VE) measured as the mean of the homozygous generations of H and L lines was 0.72. Then 51% of the variance of the F_0 population is due to genetic factors ($VGF_0 = 0.74$), and 49% to environmental effects.

The results of the selective breeding measured by the interline difference in order to reduce the effect of environmental factors are represented in Fig. 9.

The cumulated values of R or S were plotted in ordinates against the corresponding generations. The mean value of S per generation measured during the interline separation was 2.06. This selective pressure produced a progressive interline divergence at a mean rate of 0.4 per generation until F_{16} when the selection limit was reached. Then the interline difference remained constant in the following generations: $RT = 6.5 \pm 0.5$. The steady rise of cumulated S in the F_{16}–F_{19} generations was therefore entirely due to environmental effects.

The heritability of the character (h^2) measured by a least square linear regression of R/S was 0.20 ± 0.04. This is the mean value of h^2 in both lines calculated from the interline divergence.

Due to the incomplete dominance of high over low response that produced the asymmetrical effect, the h^2 was higher in L than in H line. The h^2 values calculated in each line in relation to the $\bar{x}F_0$ were 0.16 in H line and 0.26 in L line.

An attempt to calculate the number of independent loci affected by the selective breeding, made according to Equation 1, gives $n = 18$. Another estimation of n can be made considering that VGF_0 (0.74) is a maximal value of VA. This is possible since the contribution of VD to VF_0 is negligible due

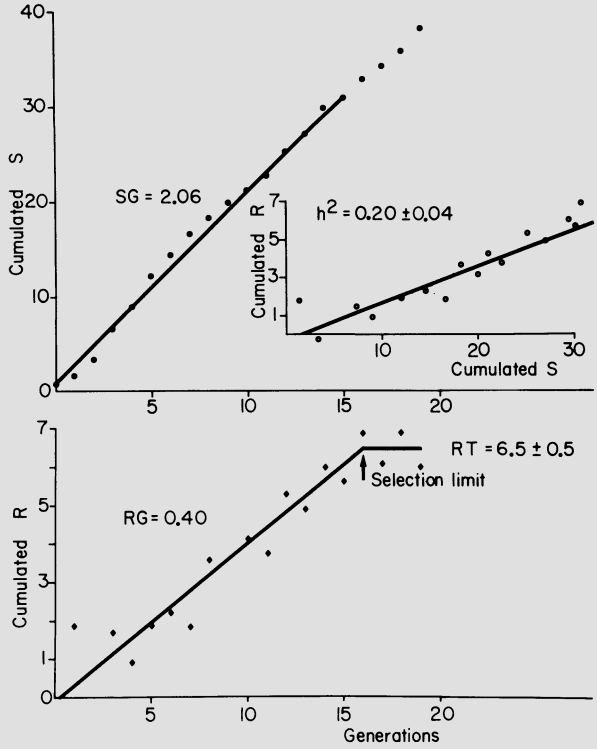

Fig. 9. Genetic analysis of Selection III — cumulated selection differential (S) log 2 in H and L lines — cumulated response to selection (R) log 2 in H and L lines. Calculation of realized heritability (h^2): plot of cumulated R on cumulated S for generations up to selection limit

to the small dominance effect. The consequent estimate of n would be 7 (Equation 9). A study of variance analysis in interline crosses analogous to that made in Selections I and II is in progress.

The data at present available indicate that the number of independent loci regulating the agglutinin responsiveness to f antigen of Salmonella is between 7 and 18.

b) Kinetics of 19S and 7S Agglutinin Production During Primary and Secondary Responses

The phenotypic character chosen for the selective breeding was the maximal secondary agglutinin response to f antigen of Salmonella. The results presented in Fig. 10 demonstrate that the group of genes separated in each line at selection limit also regulates the primary response and operates on the synthesis of both 19S and 7S antibodies.

The kinetics of primary and secondary responses to f antigen of Salm. tm. in H and L lines of Selection III (F_{16}) is represented in Fig. 10. The f agglutinin titre was established either in untreated serum or in ME treated

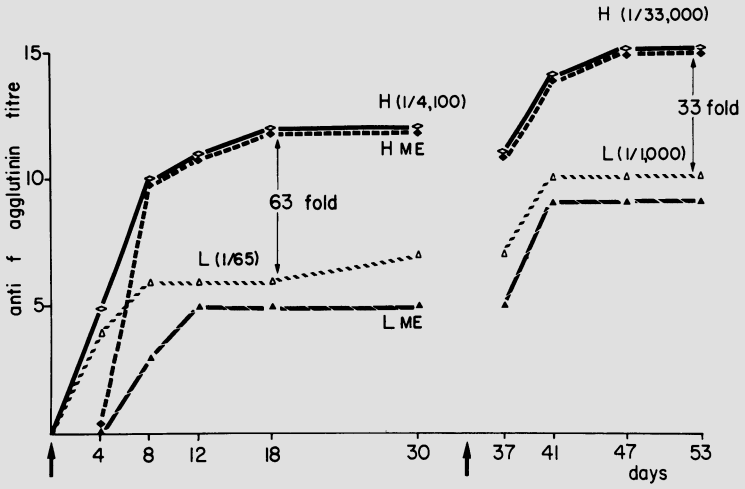

Fig. 10. Kinetics of primary and secondary responses to f antigen of Salm. in H and L lines of Selection III. Total and ME resistant agglutinin titres in groups of six mice from each line immunized i.p. with 3.3×10^8 Salm. tm. on days 0 and 32

serum in order to evaluate the contribution of ME sensitive 19S agglutinins to the total response.

The genetic status of H and L lines operates on both primary and secondary responses. The interline difference at the peak of the primary response was similar to that found for the secondary response of the selective breeding (Fig. 8).

The agglutinin titre in L line persisted at a plateau level until the 30th day, whereas in Selections I and II a rapid decline was observed (Fig. 5).

The primary and secondary responses of H line consisted essentially of ME resistant 7S antibody except during the first 4 days post primary immunization, whereas the contribution of 19S agglutinins to the total response of L line was evident after both primary and secondary immunizations.

2. Selection IV for Agglutinin Response to s Antigen of Salmonella

a) Selective Breeding and Genetic Analysis

The F_0 population was immunized with Salm. tm., then the 2 Salmonellae were alternated at each generation.

The individual titres of s agglutinins presented a normal distribution in F_0: $\bar{x} = 6.4 \pm 1.43$.

The s agglutinin response to Salm. or. measured in a population of 80 mice of the same origin as the F_0 population was $\bar{x} = 6.2 \pm 1.81$, which shows that the two Salmonellae give equivalent responses.

A small mean superiority in female responses of 0.4 was noticed but disregarded in the calculation of means and variances that were established in the total population.

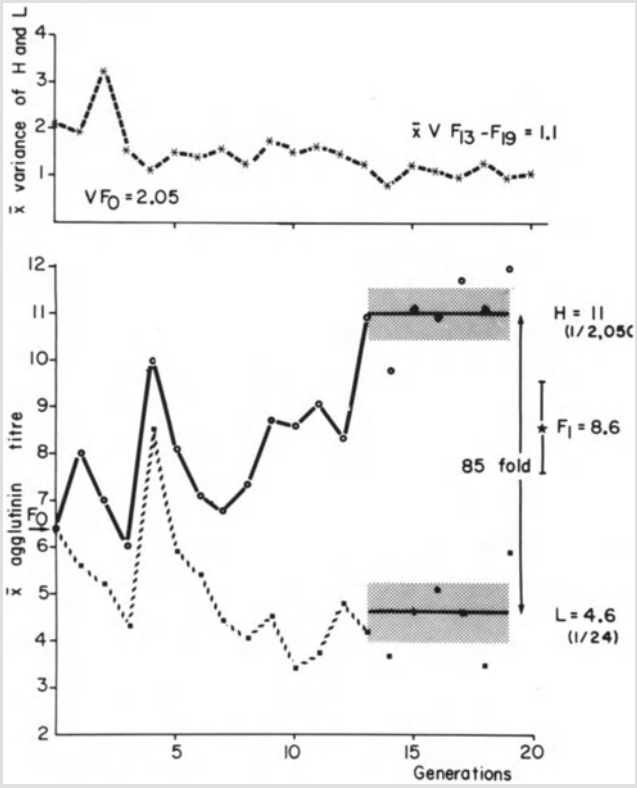

Fig. 11. Selection IV. $\bar{x}$ anti s agglutinin titres (log 2) 10 days post two i.p. injections (8 days apart) of 3.3×10^8 Salm. tm. or 10^9 Salm. or. in successive generations of H and L lines. F_0–F_{13}: Divergence of H and L lines. F_{13}–F_{18}: Total range of interline separation in homozygous generations $\pm$ standard deviation. Upper part: $\bar{x}$ of H and L variances in corresponding generations

The mean number of mice per generation was 55 ± 10 in H line and 61 ± 13 in L line. In each line eight to ten reproductive pairs were selected at each generation.

Figure 11 shows the divergence between the mean response of H and L lines produced by 19 consecutive generations of selective breeding.

The selective breeding produced a progressive interline separation accompanied by a concomitant reduction of phenotypic variance during 13 generations. Afterwards the interline separation and the value of the variances remained constant in spite of the continuation of the selection until the 19th generation. Selection limit was therefore reached in F_{13} (Fig. 12) (*Siqueira* et al., 1976).

The mean response of the seven homozygous generations at selection limit was 11.0 in H line and 4.6 in L line. The interline difference was 85-fold in terms of agglutinin titre.

The mean agglutinin response of 73 interline F_1 hybrids produced by mating F_{19} mice was 8.6 ± 0.97.

In F_1 hybrids the frequency distribution of the alleles is supposed to be 0.5, therefore the lowest mean response of the F_0 population is due to a higher frequency of bad effect alleles. The global dominance effect of high responsiveness is 25% of the additive effect: $d/a = 0.25$.

In Selection IV there was also an asymmetrical response but in the opposite direction to that observed in Selection III. In relation to the level of the F_0 population the responsiveness of H line was increased 24-fold while that of L line was decreased only 3.5-fold. In this Selection the asymmetrical response is therefore rather due to the genetic constitution of the F_0 population than to the directional dominance.

The mean environmental variance VE measured in the homozygous generations of H and L lines was 1.08. Then 47% of the phenotypic variance of the F_0 population is due to genetic factors ($VGF_0 = 0.96$) and 53% to environmental effects.

The response to selection R, and the selection differential S, calculated by the interline difference and cumulated from the F_0 population to F_{19} are represented in Fig. 12.

The two lines diverged at a mean rate of 0.48 per generation until F_{13} when the selection limit was reached. This response to selection was produced by a mean selection differential of 2.4 per generation.

The total response to selection RT measured as the mean of the seven generations at selection limit was 6.4 ± 0.5.

The heritability (h^2) calculated by R/S linear regression is 0.21 ± 0.06. Due to the asymmetrical effect the h^2 is higher in H line in which the response to selection is larger than in L line. The h^2 calculated in each line is 0.28 in H line and 0.15 in L line.

The number of independent loci, n, calculated according to Equation 1 is 12.

The minimal estimate of n obtained by postulating that the genetic variance of the F_0 population is entirely produced by additive gene effect is $n = 5$ (Equation 9).

Until the completion of the variance analysis of interline hybrids at present under way, we may conclude that the agglutinin response to s antigen of Salmonellae is regulated by a group of 5–12 independent loci.

b) Kinetics of 19 S and 7 S Agglutinin Production During Primary and Secondary Responses

The total and ME resistant agglutinin response was studied after primary and secondary immunization in H and L lines at selection limit (F_{15}). The results are presented in Fig. 13.

The difference in agglutinin titres between H and L lines was evident in both primary and secondary responses. It should be noted however that the interline difference in secondary response (8-fold) was markedly smaller than that realized at selection limit (85-fold, Fig. 11). This discrepancy may be due to the different immunization schedule used. In the experiments reported in Fig. 13 the booster injection was given 34 days post priming when a high

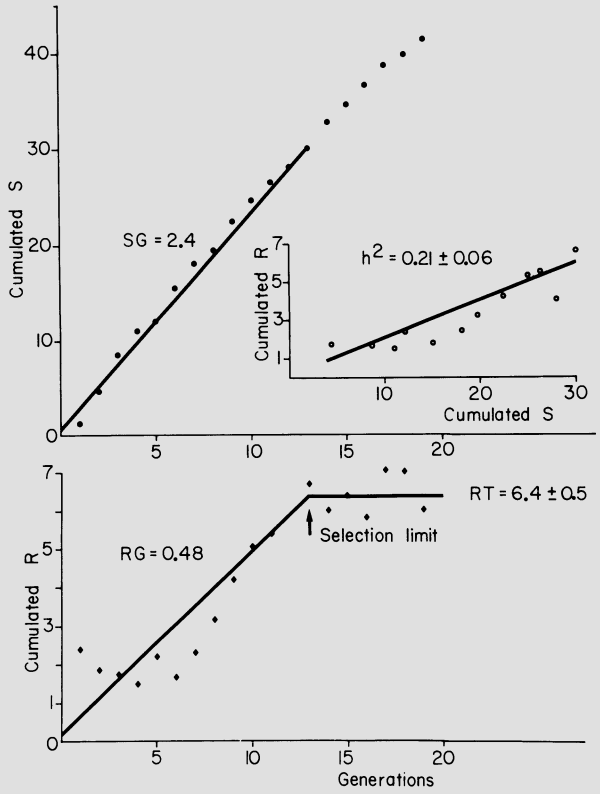

Fig. 12. Genetic analysis of Selection IV — cumulated selection differential (S) log 2, in H and L lines — cumulated response to selection (R) log 2, in H and L lines. Calculation of realized heritability (h^2): plot of cumulated R on cumulated S for generations up to selection limit

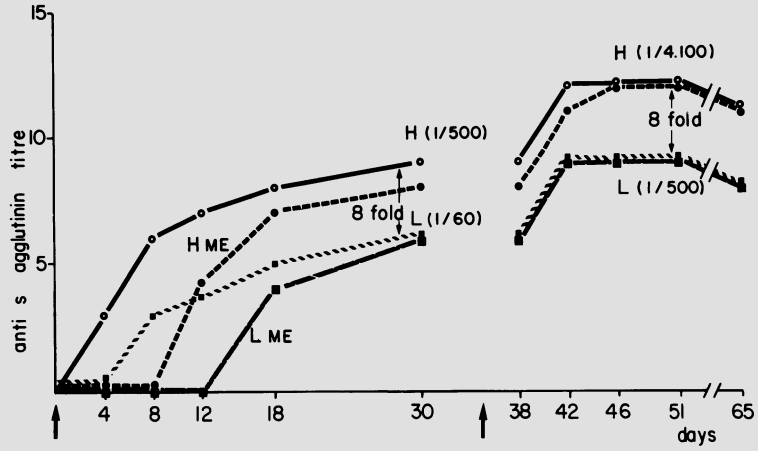

Fig. 13. Kinetics of primary and secondary responses to s antigen of Salm. in H and L lines of Selection IV. Total and ME resistant agglutinin titres in groups of six mice from each line immunized i.p. with 3.3×10^8 Salm. tm. on days 0 and 32

agglutinin level was present in the serum of both lines. In the selective breeding the two injections were only separated by an 8 days interval. At the time of the booster L mice were still unresponsive and H mice had only a low level of serum agglutinin. The regulatory effect of serum antibody on the magnitude of the secondary response may be the cause for the observed discrepancy.

During the initial phase of the primary response only 19S agglutinins were produced in both lines. The contribution of 19S antibody to the total response decreased in the advanced phase of the primary response. After secondary immunization only 7S agglutinins were synthesized in both lines.

These findings underline the importance of the environmental factors, namely the immunization procedure, on the phenotypic effect of the genes regulating immunoresponsiveness.

D. Selection V for Response to Bovine Serum Albumin (BSA) and Rabbit Gamma Globulin (RGG)

1. Selective Breeding and Genetic Analysis

These two non-cross-reacting antigens were alternated at each generation. The first ten generations of selective breeding were immunized with five doses of 2 mg alum precipitated proteins. The first injection was given intravenously, the others subcutaneously, at 5 days interval. The serum antibody titres were measured 8 days after the last injection. An increasing number of non-responder mice were found in L line during the selective breeding, therefore from F_{11} onwards, the two antigens (BSA and RGG) were administered in heat aggregated form in order to increase their immunogenicity. The F_{11} and the successive generations were immunized with two intraperitoneal injections of 1 mg heat aggregated antigens 8 days apart. The antibody response was measured 8 days after the second injection by passive hemagglutination of mouse erythrocytes coupled with BSA or RGG.

The F_0 population consisted of 73 outbred albino mice obtained from four independent colonies. The mean number of mice per generation was 52 ± 16 in H line and 53 ± 11 in L line. Six to nine breeding pairs were culled at each generation.

The mean sex effect, that is a higher responsiveness in females, measured in all the generations, was very small: 0.2 log 2. The means and variances were calculated from individual data of both sexes.

The mean response of the F_0 population immunized with BSA was 6.1 ± 3.9.

The results of the selective breeding are shown in Fig. 14 (*Passos* et al., 1977). The two lines diverged very quickly. In F_6–F_{10} responsiveness in H line rose to a plateau while that in L line remained at a very low level. In these generations many L responders were negative, preventing the correct parental culling. From F_{11} when the two antigens were administered in aggregated form the response was in fact increased to about the same extent in both lines. So, in L line the correct selection of parents was possible again. Nevertheless the interline difference was not larger in F_{11}–F_{16} than in F_6–F_{10}. This means that selection limit was already reached in F_6 (Fig. 15). The increased

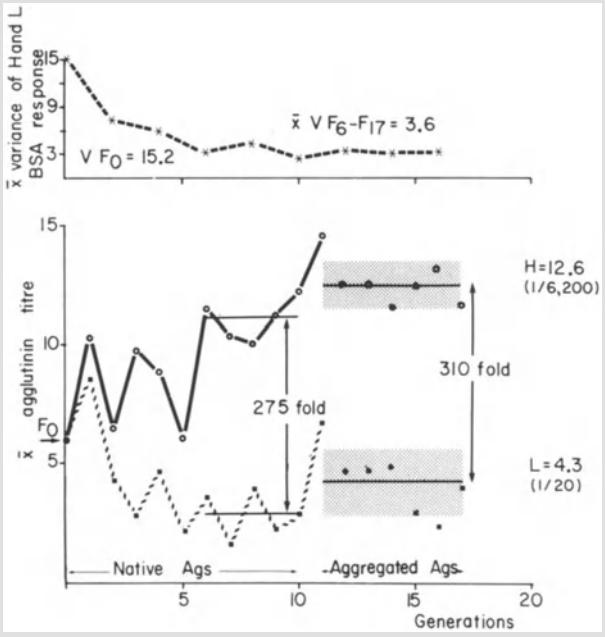

Fig. 14. Selection V. $\bar{x}$ of passive hemagglutination titres (log 2) in successive generations of H and L lines (two immunizations procedures were used, details of which are mentioned in the text). F_0–F_6: Divergence of H and L lines (native antigens). F_6–F_{10}: Total range of interline separation in homozygous generations immunized with native antigens. F_{11}–F_{17}: Total range of interline separation in homozygous generations immunized with aggregated antigens ± standard deviation. Upper part: $\bar{x}$ of variances in corresponding generations of H and L lines (immunized with BSA)

immunogenicity of heat aggregated antigens produced an environmental effect acting in both lines alike without modification of the interline difference.

In F_0–F_{10} generations (immunized with the same procedure) the selective breeding produced an asymmetrical effect. In relation to the level of the F_0 population the agglutinin response of H line was increased 32-fold while it was only decreased 9-fold in L line.

The variances of individual agglutinin titres of the generations immunized with BSA are represented in the upper part of Fig. 14. Since the variances of H and L lines are similar at each generation, the mean value is given.

The variance values decreased rapidly during the interline separation and remained constant in the homozygous generations at selection limit (F_6–F_{17}) when they are only due to environmental effects (VE = 3.6). The variance of the F_0 population immunized with BSA was 15.2, therefore 76% of VF_0 was caused by genetic factors ($VGF_0 = 11.6$) and 24% by environmental effects.

The effect of the selective breeding in terms of cumulated R and S calculated from the interline divergence is shown in Fig. 15. The mean agglutinin titres of the generations immunized with BSA and those immunized with RGG at selection limit were similar (Fig. 14). The data obtained in successive generations

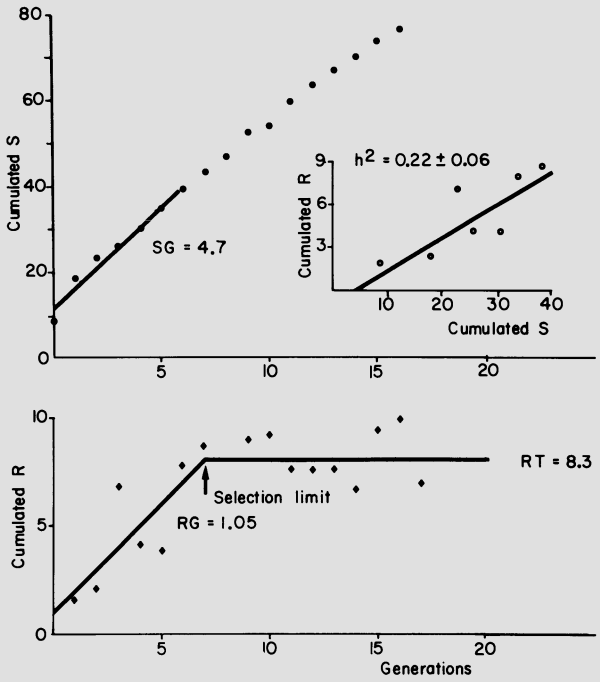

Fig. 15. Genetic analysis of Selection V — cumulated selection differential (S) log 2, in H and L lines — cumulated response to selection (R) log 2, in H and L lines. Calculation of realized heritability (h^2): plot of cumulated R on cumulated S for generations up to selection limit

can thus be directly cumulated to calculate R. Since the difference in the variances of the responses to BSA and RGG was small, the S values of successive generations were also cumulated and S represents the mean value of the genetic pressure relative to the two antigens.

The response to selection was stronger in Selection V than in the preceding four Selections. It was 1.05 per generation, so the selection limit of 8.3 was reached in F_7. This rapid response to selective breeding is due to the large phenotypic variance of the F_0 population, mainly produced by genetic factors (76%). A high selective pressure: SG = 4.7 was exerted. Consequently the heritability ($h^2 = 0.22$) is similar to that observed in the other Selections. In spite of the asymmetrical effect mentioned above (Fig. 14) the heritability is very similar in both lines: h^2 is 0.20 in H line and 0.23 in L line. Unfortunately F_1 hybrids were not tested in the same immunization condition as for the F_0–F_{10} generations, so the effect of the directional dominance on h^2 cannot be established. The mean response in F_1 immunized with heat aggregated BSA is 6.7 ± 3.3. Compared with the response of homozygous parental lines immunized in the same way, this value indicates a global dominance effect of low responsiveness ($d/a = -0.43$).

The strong response to selection produced in few generations of selective breeding and the large values of VF_0 suggest that in Selection V the immune

responses are controlled by a smaller number of loci than in the preceding Selections. In fact, according to Equation 1, only two to three independent loci should intervene. The study of antibody responses in interline crosses is in progress. At present only data obtained in a group of F_1 and F_2 interline hybrids is available. The calculation of n, according to Equations 3, 7 and 9, gives the estimate of three to four loci.

The results of these three estimations obtained from independent data are concordant. They indicate that the quantitative antibody response to the protein antigens used in Selection V is regulated by a group of two to four independent loci.

2. Kinetics of Primary and Secondary Antibody Responses to BSA and RGG

The kinetics of the antibody response to heat aggregated BSA and RGG was studied during the primary response and after a secondary challenge given 35 days later.

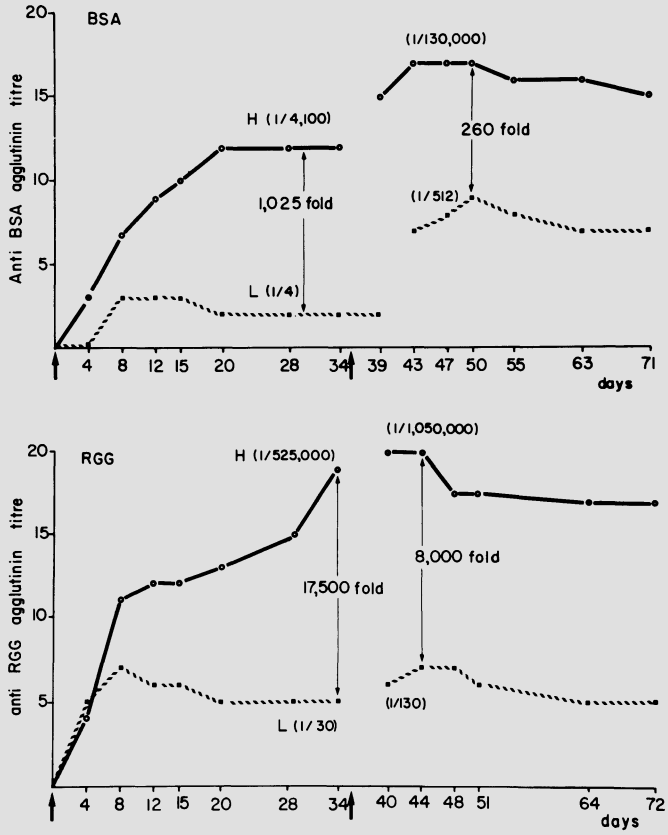

Fig. 16. Kinetics of primary and secondary responses to BSA and RGG in H and L lines of Selection V. Total agglutinin titres in groups of 15 mice from each line, immunized i.p. with 1 mg heat aggregated BSA or RGG on days 0 and 35

The general pattern of primary responses to BSA and RGG was very similar (Fig. 16). In L mice the agglutinin titre reached the peak 8 days post immunization. In H line, on the contrary, there was a progressive increase in antibody titre up to the 20th–34th day post immunization. The interline difference became therefore extremely large at the end of the primary response.

The increase of antibody level after the secondary challenge with RGG was very small compared with the pre-challenge titre, either in H or L line. On the contrary a sharp secondary response to BSA was observed in both lines.

These findings demonstrate that both primary and secondary responses were submitted to the same genetic regulation.

The interline separation obtained in the secondary responses by the immunization schedule used in the experiments reported in Fig. 16 was larger than that produced by the immunization schedule used during the selective breeding (Fig. 14). This stresses the importance of the immunization procedure on the phenotypic expression of the genes.

E. Comparison of the Results of the Five Selections

The five Selections described above were successful. Selection limit was reached after progressive divergence and homozygous H and L responder lines were obtained.

The main genetic parameters of each Selection are summarized in Table 6. These results can be compared since the scale of measurement is identical. The selection procedure was also similar, namely concerning population sizes and selection criteria.

Table 6. Comparison of the genetic analysis of the five Selections

	Selections				
	I	II	III	IV	V
Selection antigens	SE		Salm. tm. & or.		BSA
	PE	SE	f ant.	s ant.	RGG
Foundation / Population: VP	2.7	2.43	1.47	2.04	15.3
Foundation / Population: VG/VP %	63	57	52	56	76
Response to selection: RT	7.8	6.8	5.8	6.3	8.1
Response to selection: RG	0.48	0.42	0.39	0.37	1.05
h_2 [realized heritability]	0.21	0.20	0.18	0.18	0.22
Number of loci: Equation 1	15	12	16	12	3
Number of loci: Range of other estimates	9–15	2–12	7–18	5–12	2–4
Global dominance: d/a	0.27	0.54	0.20	0.25	−0.43

The differences between the five experiments were restricted to two important factors: the nature of the selection antigen and the procedure of immunization.

The characteristics of the F_0 populations are roughly similar in Selections I, II, III and IV whereas, in Selection V, the phenotypic variance is larger. This difference is essentially due to the genetic component of VF_0. This suggests the intervention of a less complex genetic control, since VF_0, like VF_2, is inversely proportional to the number of loci.

The response to selection is expressed by both RT and RG. RT has the same order of magnitude in the five Selections. This might result from a physiological homeostatic mechanism limiting gene expression. RG is similar in Selections I, II, III and IV in which the maximal interline separation is reached after 13–16 generations of selective breeding. It is clear that the high value of RG in Selection V is due to the large genetic variance of the F_0 population, therefore selection limit is reached in the seventh generation.

The most important parameter to be compared is the realized heritability (h^2) that is close to 0.20 in the five Selections. It must be stressed that the h^2 value depends on the nature of the selected character, especially in relation to fitness. As a rule, higher h^2 values are found for characters of lower adaptive importance. The constant and moderate value of h^2 in our results is understandable, since the quantitative regulation of immune responses is obviously an important component of fitness.

The number of loci responsible for interline difference is indicated first as it results from calculations made with Equation 1. The range of estimates obtained by other methods (presented in detail in the preceding paragraphs) is also reported since the calculation of n is only approximate.

In Selections I, II, III and IV, it may be considered that about ten loci are operating. As anticipated, in Selection V, the number of loci is significantly smaller.

Finally, the d/a ratio, obtained by comparing the responses of F_1 with those of the homozygous parental lines, is also mentioned. The values are positive in Selections I, II, III and IV, which indicates a global dominance effect of high responsiveness. In Selection V on the contrary, the global dominance effect is in the direction of low responsiveness.

IV. Non-Specific Effect of Selective Breeding

The effect of the five selective breedings is not restricted to the antigens used in each Selection (selection antigens). It also operates on antibody responsiveness to many natural immunogens unrelated with the selection antigens. Nevertheless this non-specific effect was not general nor equivalent for all the antigens investigated. In fact the extent of the interline difference in responsiveness to various antigens varied. Sometimes it was as large as that obtained for the selection antigens but it could be smaller for other unrelated antigens, and finally there were some antigens that induced a similar antibody response in H and L lines.

The amplitude of the non-specific effect depends on the nature of the antigens used for the selective breeding and probably also on the immunization procedure.

A. Selection I

In this Selection, the non-specific effect was almost general. The H and L mice were respectively high and low responders to practically all the antigens tested so far (*Biozzi* et al., 1974, 1975). Nevertheless two exceptions have been reported by *Howard* et al. (1974) concerning dextran and levan which induce a similar response in both lines. The two polysaccharides are thymus independent antigens inducing only 19S antibody response. These exceptions are not due to these peculiarities since the Pneumococcus polysaccharide SIII, which has the same characteristics, induces quite different responses in H and L lines (*Howard* et al., 1972). The lack of interline difference in the responses to dextran and levan has been ascribed to the similar rate of their metabolism in the macrophages of H and L responders (*Howard* et al., 1974; *Wiener* and *Bandieri,* 1974).

Apart from dextran and levan, the antibody response to all the other antigens tested was always stronger in H than in L lines whatever the method of immunization used and the adjuvant added. This was demonstrated for the following multideterminant immunogens:

Heterologous erythrocytes: Rat, Horse and Man;

Proteins: hemocyanin (*Unanue* et al., 1974), Bovine serum albumin (*Heumann* and *Stiffel,* 1978), Hen egg albumin (*Prouvost-Danon* et al., 1971);

Haptens: DNP (*Del Guercio* and *Zola,* 1972) and TNP (*Doria* et al., 1978) coupled with various carriers and picryl chloride administered by skin painting (*Mouton* et al., 1974);

Bacterial polysaccharides: Pneumococcus SIII (*Howard* et al., 1972), polysaccharides A and C Streptococcus (Eichmann, personal communication);

Histocompatibility and tumor antigens (*Liacopoulos-Briot* et al., 1972);

Viruses: T_4 bacteriophage (*Howard* et al., 1974) and influenza virus (*Floc'h* and *Werner,* 1978);

Bacteria: flagellar and somatic antigens of Salmonellae (*Sant'Anna* et al., 1979), Streptococci A and C (Eichmann, personal communication), Brucellae (*Cannat* et al., 1978), Yersinia pestis (*Dodin* et al., 1972);

Parasites: Plasmodium berghei (*Biozzi* et al., 1978), Tripanosoma cruzi (*Kierszenbaum* and *Howard,* 1976), Leishmania tropica (Howard, personal communication), Schistosoma donovani (*Blum* and *Cioli,* 1978), Trichinella spiralis (*Perrudet-Badoux* et al., 1975, 1978).

Detailed studies of responses to several unrelated antigens have been made:

1. Salmonella Typhimurium Antigens

The interline difference in agglutinin responses to flagellar (f) and somatic (s) antigens of Salmonella typhimurium (Salm. tm.) is shown in Fig. 17 (*Sant'Anna* et al., 1979).

The kinetics of primary agglutinin response to f and s antigens markedly differ in H and L lines. Thirteen days post-immunization, the interline difference in f and s agglutinin response is 250- and 25-fold respectively. For comparison with the separation obtained for the selection antigens, it should be recalled

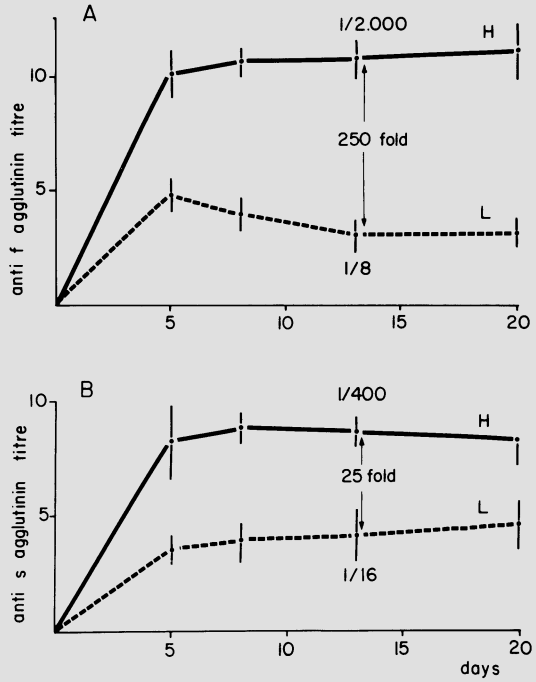

Fig. 17. Kinetics of primary response to f (A) and s (B) antigens of Salm. tm. in H and L lines of Selection I. Agglutinin titres in groups of ten mice from each line immunized i.p. with 3.3×10^8 Salm. tm.

that the interline difference in primary response to SE is 220-fold in Selection I (Fig. 1); 63-fold for the f antigen of Salm. in Selection III (Fig. 10); and 8 fold for the s antigen of Salm. in Selection IV (Fig. 13). The non-specific effect of Selection I for the responsiveness to unrelated Salmonella antigens is thus equal or larger than that concerning the selection antigens used in the three Selections.

2. Bovine Serum Albumin

Heumann and *Stiffel* (1978) demonstrated that the physical state of the antigen and the immunization procedure have an important effect on the level and kinetics of antibody response of H and L lines. An example is given in Fig. 18. Bovine serum albumin (BSA) was injected either intravenously in the form of soluble plurimolecular aggregates (CA-BSA) or intraperitoneally in the form of alum precipitates (Alum-BSA).

The intravenously injected CA-BSA was rapidly cleared from the circulation by liver and spleen macrophages. The antibody response was therefore induced in the spleen by a single antigen pulse. On the contrary, after intraperitoneal immunization, the alum precipitated BSA induced a continuous and protracted antigenic stimulation.

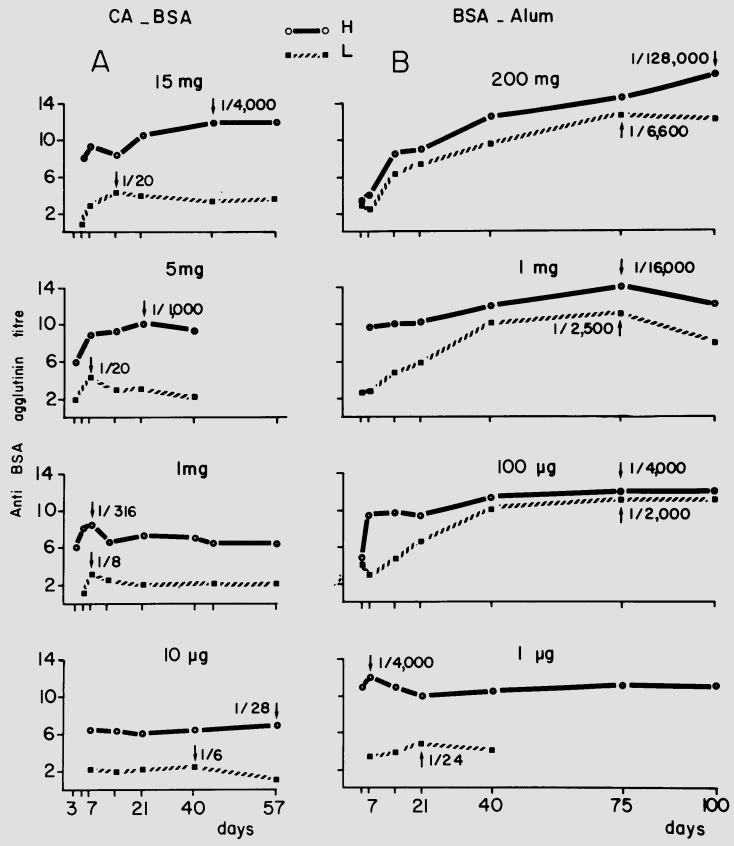

Fig. 18. Kinetics of primary response to increasing doses of BSA in H and L lines of Selection I. A. Heat aggregated BSA: CA-BSA injected i.v. B. Alum precipitated BSA injected i.p. in groups of 10–20 mice from each line

The antibody response to all doses of CA-BSA was markedly stronger in H than in L mice. For the largest antigen dose (15 mg) the interline difference was 200-fold. This non-specific effect corresponds to the interline difference observed for the optimal dose of selection antigen (SE) that is 220-fold (Fig. 1). Both SE and CA-BSA antigens injected intravenously produced a single pulse of immunogenic stimulation in the spleen.

The difference in antibody response between H and L mice immunized intraperitoneally with alum precipitated BSA was reduced, in particular in the advanced phase of the response to the largest antigen doses.

The different results obtained with the two immunization procedures can be explained by the genetic modifications of the antigen catabolism in macrophages described in Chapter V.B. The persistence of the antigen in macrophages is a limiting factor of antibody response after the single pulse of antigen produced by intravenous immunization (*Biozzi* et al., 1972a). On the contrary, this limiting factor may be partially overwhelmed when the intraperitoneal immunization with the adjuvant precipitated antigen provides a continuous antigenic supply.

Table 7. Induction of immunological memory to BSA: Secondary responses to CA-BSA in groups of H and L mice primed with increasing doses of CA-BSA

Priming dose μg	Secondary* challenge μg	Peak agglutinin titre H line	L line
—	10	360	6
0.01	10	128	—
0.1	10	16000	—
1	10	4000	32
10	10	8000	8
100	10	4000	1024

* Two months after priming

This is a typical example of the modification of the phenotypic expression of the genotype by an environmental factor.

The induction of immunological memory to BSA was also affected by the genes regulating immunoresponsiveness in H and L lines as shown in Table 7. In H line the minimal priming dose was 0.1 μg whereas a 1000-fold higher dose (100 μg) was required to induce the immunological memory in L line.

The modification of the threshold immunizing dose of selection antigen described in chapter III.A (Fig. 3) was also observed for unrelated antigens such as BSA and hen egg albumin. The minimal dose of CA-BSA giving a detectable response was 10^{-3} μg in H line and 100 μg in L line, this makes a 10000-fold difference. This effect is larger than that observed for the selection antigen which is about 100-fold (Fig. 3).

3. Hen Egg Albumin

A detailed study of antibody response to hen egg albumin (EA) was made in H and L lines and their hybrids by *Prouvost-Danon* et al. (1977). A wide range of alum precipitated EA doses was used with the emphasis on small doses since the responsiveness to limiting doses of EA is controlled, in inbred mice, by a specific Ir gene (*Vaz* et al., 1971).

The results obtained in H, L and F_1 hybrids, reported in Fig. 19, show some characteristics already observed in the study of responsiveness to other antigens: SE (Fig. 3) and CA-BSA. The sensitivity to antigen stimulation is higher in H than in L line: the threshold immunizing EA dose is about 100-fold lower in H than in L mice.

The interline difference, which was very large for low EA doses, decreased as the antigen dose was increased since the immunization was given by intraperitoneal route with alum precipitated antigen.

An important result was obtained concerning the relationship between the antigen dose and the dominance effect in the F_1 hybrids. High response character, which is dominant for the dose of 1 μg, became progressively recessive as the antigen dose decreased, and was completely recessive for the dose of 0.05 μg.

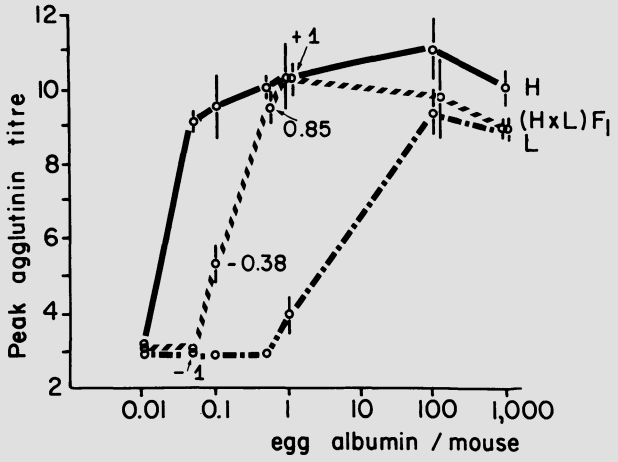

Fig. 19. Dose-response to egg albumin (EA) in H and L lines of Selection I and their F_1 hybrids. Peak values of passive hemagglutination titres in groups of 8–15 mice immunized i.p. with increasing doses of alum precipitated EA. Figures close to F_1 values are d/a values for corresponding doses

Table 8. Observed versus theoretically expected percentages of responder mice in interline hybrids immunized with 0.05 μg EA, according to a one-locus model hypothesis

	Number of mice	Responder mice (agglutinin titres > 4 log 2)			
		Observed		Expected	
		number of mice	%	number of mice	%
H	13	13	100		
L	10	2	20		
F_1	5	0	0		
F_2	46	6	13	14	30
BcH	18	11	61	9	50
BcL	5	0	0	0.5	10

The inheritance of responsiveness to the threshold EA dose of 0.05 μg, that permits a clear discrimination between responders and non-responders, was studied in F_1, F_2 and both backcrosses.

The distribution of parental phenotypes, responder or non-responder, in interline hybrids, was compared with the theoretical distribution based on a single locus hypothesis and the observed data, reported in Table 8, proved to be close to the expected ones, which demonstrates that a single locus operates. The variance analysis of larger groups of mice is in progress. The preliminary results confirm the monogenic control of responsiveness to this EA dose.

It was particularly interesting to know whether or not this single locus operating at the threshold EA dose was H-2 associated. Consequently the anti-

body responsiveness was studied in groups of F_3 H/H and F_3 L/L mice. As described in chapter III.A.3, these mice are homozygous for the H-2 phenotype of H and L mice respectively on an identical background equivalent to that of F_2 mice. The results show that all F_3 H/H mice were responders, as H mice are, whereas all F_3 L/L mice were non-responders, as L mice are (*Mouton* et al., 1977).

The paramount importance of an H-2 linked gene in the control of antibody response to threshold EA and SE (Chapter III.A) doses in H and L lines is well established. This gene participates also in the regulation of responsiveness to optimal doses of antigen but in this case its quantitative importance is reduced since the control becomes polygenic.

B. Selection II

In this Selection the non-specific effect was less extensively studied than in Selection I; nevertheless, the data summarized in Table 9 clearly indicate a concomitant modification of antibody responsiveness to five immunogens unrelated with the selection antigen (SE).

The results are expressed in terms of interline differences of log 2 agglutinin titres or of agglutinin titres ratios.

The antibody responses to all the unrelated antigens are markedly higher in H than in L line, although the difference is always smaller than that concerning the selection antigen (34%–65%).

C. Selections III and IV

In these two Selections the importance of the non-specific modification of antibody responsiveness produced by the selective breeding was studied throughout the selective process, from the F_0 population to selection limit (*Siqueira* et al., 1977). The Salmonella contains two independent antigens, f and s. In each Selection the agglutinin response was measured for the two antigens. The response to Selection, measured in terms of progressive interline difference for the selection antigen, was accompanied by an equivalent effect on the response to the associated antigen, as shown in Fig. 20.

The interline difference in the agglutinin response to the selection antigen is represented, in abscissae, against the same effect measured for the associated antigen in all the generations from the F_0 population to selection limit, in ordinates. The slope measured by a least square linear regression is 0.97 in Selection III and 0.94 in Selection IV. Thus the modification of responsiveness to the associated antigen is almost equivalent to that realized for the selection antigen. The non-specific effect is observed from the beginning of the selective breeding and increases until selection limit, which indicates that the same genes accumulated progressively in each line during the selective process regulate the antibody responsiveness to the two unrelated antigens f and s. The progressive interline separation for antibody responses to other antigens: SE, DNP-HGG and BGG measured at different stages of the selective breeding was

Table 9. Non-specific effect in Selection II — Maximal agglutinin titres in H and L mice immunized with various unrelated antigens — Comparison of interline difference with that observed for selection antigen

Antigen	Agglutinin response				p	Interline difference			Number of mice	Generation tested
	H line		L line			H–L Log 2	H/L Aggl. titre	% of Selection Ag.		
	Log 2	Aggl. titre	Log 2	Aggl. titre						
Selection Ag. Sheep erythrocytes (SE)	11.6±1.0	3100	4.9±1.2	30	<0.001	6.7	103	100	466 H 455 L	F_{14}–F_{22}
Pigeon erythrocytes (PE)	14.3±2.0	20160	9.9±1.3	955	<0.02	4.4	21	65	5 H 5 L	F_{14}
Hen egg albumin (EA)	8.1±1.0	274	4.9±0.9	30	<0.001	3.2	9	48	6 H 6 L	F_{17}
f antigen of Salm. tm.	10.4±1.6	1350	6.4±0.7	84	<0.001	4.0	16	60	8 H 8 L	F_{23}
s antigen of Salm. tm.	6.7±1.6	104	4.4±0.6	21	<0.01	2.3	5	34	8 H 8 L	F_{23}
Plasmodium berghei	13.5±0.6	11600	10.5±0.7	1450	<0.001	3.0	8	44	9 H 12 L	F_{18}

SE = 14th day after i.v. injection of 5×10^8.
PE = 14th day after i.v. injection of 1×10^8.
EA = 21st day after i.p. injection of 1 µg alum precipitated.
Salm. tm. f = 21st day after i.p. injection of 3.3×10^8 Salm. tm.
Salm. tm. s = 21st day after i.p. injection of 3.3×10^8 Salm. tm.
P. berghei = 7 days after 6 i.p. injections of 3×10^7 parasitized erythrocytes irradiated at 60000 r (immunofluorescent assay).

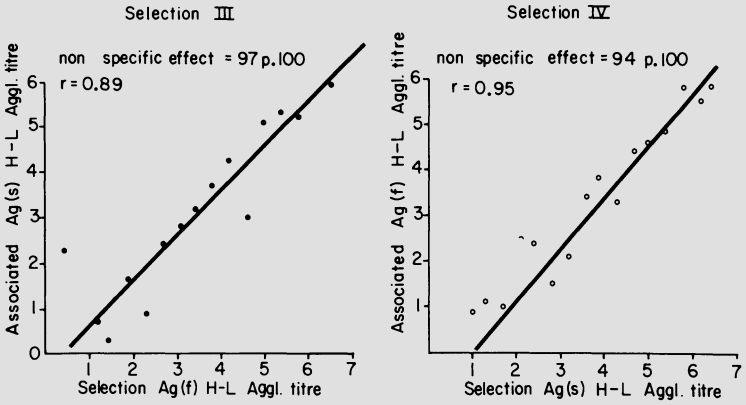

Fig. 20. Correlation between interline differences in responses to selection antigen and associated antigen in Selections III and IV. Difference between means of peak agglutinin titres to both antigens in successive generations of the two Selections (correlation calculated by a least square regression curve)

also demonstrated in Selections III and IV with a very important exception concerning Selection IV. In this Selection an equivalent agglutinin response of H and L lines to SE was demonstrated in F_4, F_6, F_8, F_{10}, F_{12} and F_{14} (*Siqueira* et al., 1977).

The antibody response to ten unrelated antigens tested in H and L lines of Selection III at selection limit is reported in Table 10 and compared with the interline difference concerning the selection antigen f considered as 100%.

The almost equivalent non-specific effect (92%) on the responsiveness to the associated antigen s observed during the interline separation (Fig. 20) was confirmed.

A very large interline difference was also observed in the agglutinin response to Salmonella anatum especially for the s antigen. In previous experiments carried out in H and L mice it was clearly demonstrated that Salm. tm. and Salm. anatum were completely antigenically unrelated in terms of immunogenic and antigenic characteristics of both f and s antigens.

Another strong non-specific effect concerns BGG, since the L line is almost completely unresponsive to this antigen. An intermediate non-specific effect is observed for the other antigens. The interline difference in antibody responses to DNP hapten coupled to BGG or HGG was noticeable, though smaller, than that obtained with the two native carriers.

A very important exception concerns the agglutinin response to pigeon erythrocytes since there was no significant difference between H and L lines.

The non-specific modification of the antibody responses to 11 unrelated antigens in H and L lines of Selection IV is represented in Table 11.

In this selection the non-specific effect was smaller and less extensive than that produced in Selections I, II and III. It was very pronounced for some antigens, like the associated f antigen and the unrelated f and s antigens of Salm. anatum, intermediate for other antigens such as BGG and BSA, small

Table 10. Non-specific effect in Selection III — Maximal agglutinin titres in H and L mice immunized with various unrelated antigens — Comparison of interline difference with that observed for selection antigen

Antigen	Agglutinin response				p	Interline difference			Number of mice	Generation tested
	H line		L line			H–L Log 2	H/L Aggl. titre	% of Selection Ag.		
	Log 2	Aggl. titre	Log 2	Aggl. titre						
Selection Ag. f antigen Salm. tm. and Salm. or.	12.7±0.8	6650	6.2±0.9	73	<0.001	6.5	91	100	236 H 205 L	F_{16}–F_{19}
Associated Ag. s antigen Salm. tm. and Salm. or.	10.8±1.1	1780	4.8±1.3	27	<0.001	6.0	66	92	236 H 205 L	F_{16}–F_{19}
s antigen of Salm. anatum	9.3±1.7	630	2.4±1.0	5	<0.001	6.9	126	106	15 H 15 L	F_{16}
f antigen of Salm. anatum	11.6±1.0	3100	7.1±0.8	140	<0.001	4.5	22	69	15 H 15 L	F_{16}
Bovine gamma globulin (BGG)	8.9±2.3	477	2.9±2.1	7	<0.001	6.0	68	92	16 H 16 L	F_{18}
DNP_{22}–BGG	11.7±1.3	3330	7.6±0.6	193	<0.001	4.1	17	63	14 H 14 L	F_{16}
Bovine serum albumin (BSA)	8.8±2.2	445	4.1±1.0	17	<0.001	4.7	26	72	15 H 15 L	F_{18}
Human gamma globulin (HGG)	9.5±1.9	720	5.6±1.4	48	<0.001	3.9	15	60	15 H 15 L	F_{16}
DNP_{27}–HGG	10.6±1.4	1550	7.8±0.8	220	<0.001	2.8	7	43	15 H 15 L	F_{16}
Sheep erythrocytes (SE)	11.6±1.5	3100	7.9±1.2	238	<0.001	3.7	13	57	16 H 16 L	F_{16}
Pigeon erythrocytes (PE)	9.7±1.7	830	9.1±1.0	548	n.s.	0.6	1.5	9	15 H 15 L	F_{16}

Salm. = 10–12 days after 2 i.p. injections (8 days apart) of 3.3×10^8 Salm. tm. or 10^9 Salm. or. and Salm. anatum.

BGG, HGG = 10 days after the third injection of 1 mg alum precipitated BGG or HGG the first given i.v. the two others i.p. on days 0, 4 and 8.

DNP–HGG, DNP–BGG = 12 days after i.p. injection of 1 mg in CFA.

BSA = 10 days after two i.p. injections (8 days apart) of 1 mg (heat aggregated).

SE = 12 days after i.v. injection of 5×10^8.

PE = 13 days after i.v. injection of 1×10^8.

Table 11. Non-specific effect in Selection IV — Maximal agglutinin titres in H and L mice immunized with various unrelated antigens — Comparison of interline difference with that observed for selection antigen

Antigen	Agglutinin response				p	Interline difference			Number of mice	Generation tested
	H line		L line			H–L Log 2	H/L Aggl. titre	% of Selection Ag.		
	Log 2	Aggl. titre	Log 2	Aggl. titre						
Selection Ag. s antigen Salm. tm. and Salm. or.	11.0±1.0	2048	4.6±1.2	24	<0.001	6.4	85	100	352 H 361 L	F_{13}–F_{19}
Associated Ag. f antigen Salm. tm. and Salm. or.	12.7±1.0	6650	7.0±1.1	128	<0.001	5.7	52	89	352 H 361 L	F_{13}–F_{19}
DNP Salm. tm.	14.7±0.8	26600	9.8±2.9	890	<0.01	4.9	30	76	6 H 6 L	F_{15}
f antigen Salm. anatum	12.4±0.9	5400	7.0±0.7	128	<0.001	5.4	42	84	16 H 16 L	F_{18}
s antigen Salm. anatum	9.7±1.4	830	5.1±1.5	34	<0.001	4.6	24	72	16 H 16 L	F_{18}
Bovine gamma globulin (BGG)	6.7±2.4	104	3.5±1.9	11	<0.02	3.2	9	50	12 H 12 L	F_{16}
Bovine serum albumin (BSA)	9.1±2.7	550	6.2±2.4	73	<0.05	2.9	8	45	15 H 17 L	F_{18}
Pigeon erythrocytes (PE)	10.4±2.0	1350	8.9±1.6	480	<0.05	1.5	3	23	14 H 15 L	F_{21}
DNP_{27}–HGG	10.9±0.5	910	10.2±0.8	1176	=0.01	0.7	1.6	11	14 H 14 L	F_{16}
Human gamma globulin (HGG)	5.2±1.5	37	4.7±1.3	26	n.s.	0.5	1.4	8	14 H 16 L	F_{18}
Sheep erythrocytes (SE)	9.1±1.1	550	10.1±0.7	1100	=0.01	−1	0.5	−15	14 H 14 L	F_{16}
Human erythrocytes (HE)	5.8±2.1	55	6.9±2.0	120	n.s.	1.1	0.46	−17	11 H 11 L	F_{18}

Salm. = 10–12 days after 2 i.p. injections (8 days apart) of 3.3×10^8 Salm. tm. or 1.10^9 Salm. or. and Salm. anatum.

DNP–Salm. = 12 days after 2 i.p. injections (10 days apart) of 5×10^8.

BGG, HGG = 10 days after the third injection of 1 mg alum precipitated BGG or HGG, the first given i.v., the other two i.p. on days 0, 4 and 8.

DNP–HGG = 12 days after i.p. injection of 1 mg in CFA.

BSA = 10 days after 2 i.p. injections (8 days apart) of 1 mg (heat aggregated).

SE = 12 days after i.v. injection of 5×10^8.

PE = 13 days after i.v. injection of 1×10^8.

HE = 8 days after i.v. injection of 1×10^8.

but significant for PE and DNP hapten coupled to Human gamma globulin (HGG). No significant difference between H and L lines was observed in the antibody responses to Human erythrocytes (HE) and HGG. Moreover the agglutinin response to SE was slightly but significantly stronger in L than in H line.

D. Selection V

The non-specific effect of Selection V was studied by immunizing H and L mice with five immunogens unrelated with the selection antigens, as indicated in Table 12.

The antibody responses of each line to the selection antigens BSA and RGG in the F_1–F_{17} generations were similar, therefore the interline difference in the antibody responses to the unrelated antigens mentioned in Table 12 are compared to the mean of the interline separation for BSA and RGG responses considered as 100%.

In preliminary experiments, it was proved that chicken gamma globulin (CGG) is immunogenically and antigenically completely unrelated with RGG. An important non-specific effect was observed in the response of H and L mice to this antigen. The interline difference was very close to that concerning the selection antigens. A small but significant non-specific effect was also observed for the antibody response to SE and f antigen of Salm. tm., but this effect was only about 13% of that found for the selection antigens. The responsiveness to s antigen of Salm. tm. and to EA was equal in H and L lines.

Results in Table 12 show that in Selection V a strong non-specific effect is only observed for one antigen out of the five so far studied.

The comparison of the results obtained in the five Selections described above indicates that the non-specific effect of the relevant genes of each selective breeding differs in both quantitative and qualitative expressions. It seems to be very broad and efficient in Selections I and III, limited and weak in Selection V, and intermediate in the other Selections (II and IV).

These differences are not yet clearly understood. They most probably depend on the complexity of the selection antigen or the immunization procedure used rather than on the difference in the genetic properties of the F_0 populations. In fact Selections III and IV are founded on the same F_0 population and Selection V on a F_0 population of the same origin as that of Selections III and IV. A plausible hypothesis, deriving from the experimental results is that the extent of the non-specific effect depends on the number of independent loci involved in each selective breeding. When a large number of loci are concerned, as for Selection I ($n=10$) and Selection III ($n=7$–18) the non-specific effect is broad, whereas for Selection V in which only three to four loci are concerned, the non-specific effect is reduced.

It may therefore be hypothesized that the number of genes required for the regulation of antibody responsiveness is inversely proportional to the antigenic complexity of the immunogen. This explains why a single specific Ir gene controls the response to synthetic polypeptides of limited molecular complexity (*Benacerraf* and *McDevitt,* 1972).

Table 12. Non-specific effect in Selection V — Maximal agglutinin titres in H and L mice immunized with various unrelated antigens — Comparison of interline difference with that observed for selection antigen

Antigen	Agglutinin response				p	Interline difference			Number of mice	Generation tested
	H line		L line			H–L Log 2	H/L Aggl. titre	% of Selection ag.		
	Log 2	Aggl. titre	Log 2	Aggl. titre						
Selection antigens										
Bovine serum albumin (BSA)	12.8±1.9	7130	4.6±2.4	24	<0.001	8.2	297		202 H	
						8.3	317	100		F_{11}–F_{17}
Rabbit gamma globulin (RGG)	12.4±2.1	5400	4.0±1.9	16	<0.001	8.4	337		214 L	
Chicken gamma globulin (CGG)	13.8±1.4	14260	6.1±2.1	68	<0.001	7.7	209	93	15 H 14 L	F_{17}
Sheep erythrocytes (SE)	9.4±0.8	675	8.4±1.1	337	<0.02	1.0	2	12	21 H 18 L	F_{15}
f antigen of Salm. tm.	10.8±0.9	1780	9.7±0.8	830	=0.001	1.1	2.1	13	15 H 15 L	F_{15}
Hen egg albumin (EA)	16.4±5.1	86450	15.2±4.0	37630	n.s.	1.2	2.3	14	14 H 14 L	F_{16}
s antigen of Salm. tm.	5.6±1.1	48	5.6±0.9	48	n.s.	0	1	0	15 H 15 L	F_{15}

BSA, RGG, CGG = 8–10 days after two i.p. injections (8 days apart) of 1 mg (heat aggregated).

SE = 12 days after 5×10^8.

Salm. tm. = 10–12 days after 2 i.p. injections (8 days apart) of 3.3×10^8.

EA = 8 days after 5 injections of 2 mg (alum precipitated), the first given i.v., the others i.p., 5 days apart.

V. Cellular Expression and Functions of the Genes Regulating the Quantitative Antibody Response

The findings reported in this chapter were obtained in mice of Selection I, the most extensively studied Selection.

The group of genes accumulated in each line by selective breeding regulates the activity of the immunocompetent cells themselves, namely lmyphocytes and macrophages. The results of the in vitro response reported later, exclude the relevant intervention of systemic mechanisms of regulation outside the lymphoid tissue.

A. Phenotypic Expression at Lymphocyte Level

The genetic modification of lymphocyte potentiality was demonstrated in vivo by adoptive immunization experiments.

Irradiated immuno-suppressed random bred mice or $(H \times L)F_1$ hybrids were restored with an equivalent number of spleen cells or lymph node cells from H or L donors and then immunized with SE. The SE agglutinin response was always much stronger in mice restored by H line cells than by L line cells. Similar results were obtained with lymphocyte populations deprived of macrophages by surface adherence on a glass beads column (*Biozzi* et al., 1974).

These results demonstrate the different potentialities of lymphocytes of H and L line mice. Similar observations have been made in mice of Selection III (M. Siqueira, personal communication).

A criticism that may be made of this type of experiments is that a graft-versus-host reaction occurs concomitantly with adoptive response. However, since the T cell mediated response is similar in both lines (paragraph E) this criticism does not contradict the essential significance of these experiments that is furthermore confirmed by the results of in vitro immunization (paragraph D).

The finding that response to thymus independent antigens such as Pneumococcus polysaccharide SIII is stronger in H than in L mice indicates that B lymphocytes are concerned by the genetic control (*Howard* et al., 1972). The possible regulatory helper or suppressor effect of T lymphocytes remains to be investigated.

B. Phenotypic Expression at Macrophage Level

The principal functions of macrophages are: antigen phagocytosis, antigen processing and participation in the mechanism of cell interactions required for antibody response.

The phagocytic function of macrophages was not affected by the selective breeding. The rate of phagocytosis of carbon particles and of Cr^{51} labelled SE by the liver and spleen macrophages is similar in H and L lines. The same amount of SE was phagocytized by spleen macrophages of both lines

2 hours after the injection of this antigen by intravenous route (*Mouton* et al., 1976). On the contrary, the rate of intracellular catabolism of phagocytized SE was rapid in L macrophages and slow in H macrophages.

The persistance of SE in immunogenic form in spleen macrophages was studied as follows: Primed random bred mice were immunized with homogenized and irradiated spleens removed from either H or L mice at various times after immunization with SE by intravenous route. The persistance of SE antigen in spleen was revealed by the agglutinin response of the recipients. Similar agglutinin titres were obtained when the spleens were removed 2 hours post SE injection. After 4 days the SE antigen was no longer detectable in L line spleens whereas it persisted for 2 weeks in H mice spleens (*Biozzi* et al., 1974). This finding was confirmed by the measure of the rate of Cr^{51} release from macrophages fed in vitro with equivalent numbers of Cr^{51} labelled SE (*Mouton* et al., 1976).

Another proof of the genetic modification of the macrophage function resulting from the selective breeding is given by the following in vivo experiment. The same number of spleen cells from F_1 hybrids was injected in X-ray immunosuppressed H or L recipients that were then immunized with SE. The SE agglutinin response was 1/1280 in H recipients and 1/80 in L recipients. This difference underlines the importance of the radioresistant macrophage functions in the antibody responsiveness of H and L mice (*Biozzi* et al., 1974).

Similar results were obtained by *Wiener* and *Bandieri* (1974) with another antigen: keyhole limpet hemocyanin. They observed that the amount of antigen bound to the membrane of L mice macrophages decreased rapidly while it persisted for a long time on the membrane of H mice macrophages. Furthermore the lysosomal enzyme activity was found to be higher in L than in H mice macrophages.

The difference in the enzyme equipment of H and L mice macrophages has a very marked influence on the survival and multiplication of ingested micro-organisms. The bactericidal activity is much stronger in L than in H mice macrophages. This difference was demonstrated for a variety of micro-organisms such as: T_4 bacteriophage (*Howard* et al., 1974), Salmonella, Lysteria monocytogenes (Fauve, personal communication), Mycobacterium tuberculosis and Mycobacterium leprae murium (Lagrange, personal communication), Brucella suis (*Cannat* et al., 1978) and Leishmania tropica (Howard, personal communication).

All these observations demonstrate that the metabolic activity of macrophages is an important physiologic parameter in the regulation of antibody responsiveness. This is in agreement with a recent report by *Ishizaka* et al. (1978). In H and L lines, this regulation modifies the persistance of the immunogenic form of the antigen in the macrophages. A slow destruction in H line macrophages induces a long lasting stimulation of the lymphocytes and then a high antibody response. The rapid antigen breakdown in the L mice macrophages is an important cause of their low antibody response. The inverse relationship between macrophage activity and antibody responsiveness is very important, to understand the mechanism of the anti-infection immunity described in Chapter VI.

C. Cytodynamics of the Immune Response

The early exponential phase of immune response after intravenous immunization with SE was studied at cellular level by determining the number of plaque forming cells (PFC) and Rosette forming cells (RFC) in the spleens of H and L mice (*Biozzi* et al., 1971, 1972a). The principal conclusions are the following:

— The difference between H and L responders is mainly due to the number of antibody forming cells, rather than to the amount of antibody released by each single cell.

— The rate of multiplication and differentiation of the clones of specific cells induced by antigen stimulation is very different in the two lines: the mean doubling time of RFC is 9 hours in H line and 16 hours in L line.

— The target cells for SE are small lymphocytes. Their number is similar in H and L lines (about 4000). The differentiation rate of this population of small lymphocytes into blast cells and finally into plasmocytes is much more rapid in H than in L mice. At the end of the exponential phase (4th day) there are 650000 RF plasmocytes per spleen in H responder mice and only 15400 in L responder mice. The H/L ratio of RF plasmocytes per spleen is identical to that of serum agglutinin titres on the same day (*Biozzi* et al., 1972b).

Other important immuno-biological characteristics of H and L lines of Selection I are the following:

— The difference in serum antibody levels between H and L responders is due to the genetic modification of the rate of antibody synthesis, whereas the metabolic decay of antibody molecules is similar in both lines (*Oriol* et al., 1972).

— The group of genes accumulated in the two lines by selective breeding, regulates the synthesis of all the classes of immunoglobulins: IgM, IgG (Fig. 5), IgG_1, IgG_2 (*Biozzi* et al., 1970; *Lieberman* et al., 1972), Reagins (*Prouvost-Danon* et al., 1971, 1977) and IgA (*André* et al., 1977).

— The total serum concentration of Ig is lower in L than in H line. This difference increases markedly after immunization and concerns all the Ig classes, as shown in Table 13.

Table 13. Levels of classes and sub-classes of immunoglobulins in H and L mice of Selection I before immunization or 14 days after i.v. injection of 5×10^8 SE

	Serum immunoglobulins (mg/ml)			
	Before immunization		After immunization	
	H line	L line	H line	L line
IgM	0.17	0.14	0.40	0.14
IgG1	0.78	0.31	3.60	0.24
IgG2	1.45	0.61	6.00	0.75
IgGA	0.54	0.34	0.90	0.46
Total	2.94	1.40	10.90	1.59

On the whole, L mice are genetically hypoglobulinemic. This explains their poor general antibody responsiveness to all antigens (*Biozzi* et al., 1970).

D. In Vitro Immune Response

The following experiments were carried out by *Doria* et al. (1978).

The antibody response was measured as the number of direct Plaque Forming Cells (PFC) obtained in spleen cell cultures immunized in vitro with SE according to the Mishell and Dutton method (*Mishell* and *Dutton*, 1967).

In each experiment, cells of H and L lines were simultaneously tested in order to eliminate the effect of the large variations that are usually observed from one in vitro experiment to another.

The results show that the response of H spleen cells were consistently and markedly higher than that of L spleen cells. The maximal interline difference was obtained on the 4th day of culture.

For the whole range of antigen doses investigated, the number of PFC was about 100-fold higher in H than in L spleen cell cultures (Fig. 21). This represents an interline difference approaching that observed after in vivo immunization. These results indicate that the genetic difference between H and L responders concerns the immunocompetent cells themselves, thus excluding the intervention of hormonal or any other mechanism of immune regulation originating from the rest of the organism.

The immunocompetent cells belong to two cell types: lymphocytes and macrophages, both required to obtain an in vitro immune response, as shown in Table 14.

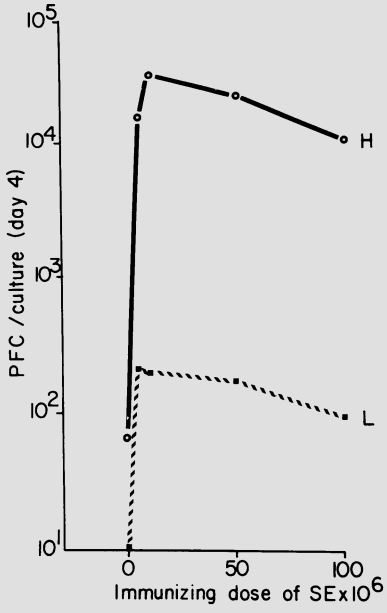

Fig. 21. In vitro responses of unseparated spleen cells cultures of H and L lines from Selection I (Mishell and Dutton culture system). PFC/culture on the 4th day post immunization with increasing doses of SE. (From *Doria* et al., 1978)

Table 14. In vitro anti SE antibody synthesis by 10^7 H or L (Selection I) spleen cells immunized in vitro with 10^7 SE

Cells in culture	ME[a]	PFC/culture (day 4)[b]	
		H	L
Unseparated	–	33000	210
	+	29800	2085
Non-adherent[c]	–	260	0
	+	24300	760

From *Doria* et al., 1978.
[a] ME added in the culture at a final concentration of 5×10^{-5}.
[b] PFC = Plaque forming cells on the 4th day of Mishell and Dutton culture.
[c] Separated by the method described by *Mosier* (1967).

Table 15. In vitro anti SE antibody synthesis by recombined H or L macrophages (1.5×10^7) and lymphocytes (10^7)

Lymphocytes versus macrophages					Macrophages versus lymphocytes				
Lymph	+	Macroph	PFC[a]	Ratio	Macroph	+	Lymph	PFC	Ratio
H		H	29700	11.2	H		H	29700	2.4
L		H	2640		L		H	12200	
H		L	12200	25.2	H		L	2640	5.4
L		L	484		L		L	484	
H		–	3110	222	H		–	4190	72
L		–	14		L		–	58	

From *Doria* et al., 1978.
PFC/culture of unseparated cells (1.5×10^7): H = 22600; L = 60.
[a] PFC: Plaque forming cells on the 4th day of Mishell and Dutton culture (immunization 10^7 SE).

The removal of macrophages, by plastic adherence (*Mosier,* 1967), suppressed, in both lines, the immune response of spleen cell populations.

The addition of ME to the cultures somehow replaced the macrophage function, restoring responsiveness in these depleted cell populations.

An attempt to measure, in H and L lines, the respective importance of lymphocytes and macrophages in the production of in vitro immune response was made by cell separation and recombination between the two lines. The results are summarized in Table 15.

As previously reported, the response of non-adherent cells was markedly reduced in comparison with that of mixed populations of both cell types. The small response obtained in separated cell cultures of H line only, resulted from a residual contamination of one cell type by the other.

The role of lymphocytes versus macrophages was measured by recombining H and L macrophages with either H or L lymphocytes. The difference in PFC attributable to lymphocyte origin was about 18-fold (11–25). The role of macro-

phages versus lymphocytes was evaluated by recombining H and L lymphocytes with either H or L macrophages, the difference in PFC attributable to macrophage origin was about 4-fold (2.4–5.4).

The results of cell recombination experiments indicate that the genetic difference between H and L responders is phenotypically expressed at the level of both lymphocytes and macrophages. The role played by lymphocytes in the in vitro immune response to SE was 4- or 5-fold more important than that played by macrophages. These results are consistent with those obtained in vivo (Paragraph A and B).

The non-specific effect of Selection I on the antibody response to other antigens (Chapter IV) was also demonstrated in in vitro experiments.

A 10-fold difference in favour of H line was observed in the in vitro response to trinitrophenyl (TNP) coupled with the T independent lipopolysaccharide from E. coli. These results, as well as those obtained in vivo with the Pneumococcus polysaccharide SIII, another T independent antigen, point out the genetic modification of B lymphocytes.

The helper effect of T cells of H and L lines was studied in vitro by adding an increasing number of in vivo carrier primed irradiated cells to F_1 spleen cells, and then by measuring the response to the hapten after in vitro immunization. The results showed that spleen cells from H or L mice displayed the same helper effect on the immune response of carrier primed cells in vitro. These experiments, described in detail by *Doria* et al. (1978) indicate that the genetic modification at the level of lymphocytes does not affect the helper function of T cells. A possible effect on the suppressor T cell function remains to be investigated.

E. Dissociation Between the Genetic Control of Humoral and Cell Mediated Immune Response

Perhaps the most important finding arising from the results of Selection I is that H and L lines, which differ so much in their general antibody responsiveness, have a similar ability for T cell mediated immunity.

The best demonstration of this fact was given by experiments of skin graft exchange between the two lines. Skin graft rejection is essentially a T cell mediated phenomenon.

There is a strong histoincompatibility between H and L mice since they differ at the H-2 locus (Chapter III.A.3). Skin grafts exchanged between the two lines are rapidly rejected. The mean rejection time of H skin grafts by L recipients was 10.4 ± 2.4 days and that of L skin grafts by H recipients was 12.6 ± 2.9 days. After skin graft rejection the serum titre of anti H-2 cytotoxic antibody was 1/110 in H line recipients and 1/3 in L line recipients. In fact, the mean survival time of skin allografts was significantly shorter in L than in H recipients ($p < 0.01$). This may be due either to the effect of facilitating antibody produced in larger amount in H line, or to a stronger participation of the very active macrophages of L line in the mechanism of skin graft rejection (*Liacopoulos-Briot* et al., 1972).

The selective breeding for SE responsiveness has therefore greatly modified the antibody response to histocompatibility antigens without changing the cellular immunity responsible for skin graft rejection.

Other T cell mediated reactions such as graft versus host reaction (*Byfield* and *Howard*, 1972), skin delayed hypersensitivity (*Mouton* et al., 1974), and in vitro response of T lymphocytes to phytohemagglutinin (*Liacopoulos-Briot* et al., 1974) have the same intensity in H and L mice.

All these findings converge in the clear demonstration that the genes regulating quantitative antibody response do not operate on cell mediated immunity. Antigen handling by macrophages seems to be required for induction of both antibody response and cell mediated immunity. The dissociation of these two phenomena in H and L lines may be explained according to two hypotheses. The pathway of antigen metabolism is different for antibody production and for cell mediated response (qualitative hypothesis). The quantitative hypothesis seems more probable. It is well known that the amount of antigen required for induction of cellular immunity is markedly smaller than that required for antibody response. A sufficient antigenic stimulation would be provided even by L line macrophages for the induction of cell mediated immunity. The shortage of immunogenic stimulation would therefore, in L mice, be a limiting factor for antibody response only.

In the literature several reports, mentioned later, indicate that cell mediated immunity is also submitted to genetic control. We may therefore conclude that the two fundamental functions of specific immunity: humoral and cellular functions, have an independent genetic regulation. This finding, together with the inverse relationship between antibody response and macrophage activity, demonstrated in a preceding chapter, constitutes the bases for the theory proposed to explain the multidirectional efficiency of the immunity considered as a defense system at the level of a genetically polymorph population (Chapter VI).

VI. Relationship Between Genetic Regulation of Immunoresponsiveness and Resistance to Aggression

As mentioned in the introduction, defence against aggression is based on the non-specific immunity mediated by macrophages and other phagocytic cells, and the specific cellular and humoral immunity mediated respectively by T and B cells. The two specific functions keep the memory of preceding stimulations. Therefore they chiefly intervene in the increased resistance induced by antecedent fortuitous (endemic) or intentionally induced (vaccination) contacts with pathogens.

Each type of infections is characterized by a peculiar aggressive device which is more efficiently contented by one or the other of the three immunity functions.

The results obtained in Selection I clearly demonstrate that each immunity function is submitted to an independent genetic regulation. The selective breeding greatly modifies the antibody responsiveness without changing the cell mediated immunity. Moreover an inverse relationship is observed between macrophage

activity and antibody responsiveness. Individuals genetically endowed with active macrophages are poor antibody responders and vice-versa. This inverse relationship is produced by the genetic modification of enzymatic antigen handling inside the macrophages of H and L antibody responders. This modification is responsible for the different bactericidal activity of macrophages when living micro-organisms are phagocytized. An individual with active macrophages will survive an infection to which the resistance is phagocytosis dependent, but will be killed by an infection efficiently coped with by antibodies and vice-versa.

The third defence mechanism is constituted by the cell mediated immunity. This response is also submitted to genetic control (*Chase,* 1941; *Benacerraf* and *McDevitt,* 1972; *Miller* et al., 1976). A major histocompatibility complex linked Ir gene is of paramount importance in this control but other independent genes also intervene in the quantitative regulation of T cell mediated responses (*Shultz* and *Bailey,* 1975; *Lubet* and *Kettman,* 1978). It is therefore very probable that cell mediated immunity to complex immunogens such as bacteria and viruses is submitted to quantitative polygenic regulation. The phenotypes of this character in a genetically heterogeneous population will therefore present a normal frequency distribution. A recent and very interesting report by *Fachet* and *Ando* (1978) demonstrates an inverse quantitative regulation of cell mediated and humoral responses analogous to our findings concerning macrophage activity and antibody responsiveness (Chapter V.B).

The independent and polygenic regulation of the three fundamental immunity functions constitutes the genetic arrangement providing the optimal defence of a genetically polymorphic population against all types of mild endemic and severe epidemic infections. In fact the largest number of individuals whose phenotypes are close to the mode of each immunity parameter will have the best multidirectional defence against all endemic infections. Therefore the genetic polymorphism of the population will be maintained by stabilizing natural selection. When the population is threatened by a severe epidemic burst the extreme phenotypes of the relevant immunity parameter will survive. As a consequence a number of individuals, inversely proportional to the epidemic severity, will resist each type of epidemic, thus ensuring the population survival.

The natural history of a population results from its confrontation with different types of epidemic infections that are coped with by one or the other of the immunity functions. Therefore directional natural selection will not appreciably modify the modal phenotype distribution resulting from polymorphism unless the population is submitted to recurrent epidemic infections of the same type.

These considerations may be applied to both natural resistance to infections and protective effect of vaccination.

The essential immunologic characteristics of H and L lines of mice of Selection I, schematized in Table 16, permit the identification of the mechanism responsible for natural or vaccination induced resistance against the different types of infection.

When untreated or vaccinated H and L mice are challenged with various infections, three outcomes may be anticipated according to the type of anti-infection defence implicated.

Table 16. Schematic representation of the immune characteristics of H and L mice of Selection I

	Humoral immunity response	Cellular immunity response	Microbicidal effect of macrophages
H line	+++	++	+
L line	+	++	+++

1. If antibodies play an important role, H mice are expected to be more resistant than L mice.

2. If macrophage activity is determinant, L mice are expected to be more resistant than H mice.

3. If cell mediated immunity plays the essential defensive role, equal resistance is likely to be found in both lines.

This last outcome may be occasionally masked by a higher production of facilitating antibody in H line. This possibility can be experimentally tested by passive immunization of L recipients with H line immuneserum.

Of course, cooperation or antagonism may exist between the three immunity mechanisms. The final issue resulting from their balance will depend on the limiting parameter.

The three possible outcomes have been verified in different types of infection (*Biozzi* et al., 1978). Some typical results are now briefly presented.

A. Anti-Infection Immunity

1. Macrophage Dependent Immunity

Gram negative micro-organisms such as Salmonella, Brucella, Yersinia, Mycobacteria, are "intracellular parasites". They are easily phagocytized by the macrophages but can survive and multiply inside the phagocytic cells. The antibodies are inefficient against this type of infection.

The specific or non-specific stimulation of macrophages activity produces an efficient protection against Salmonella infections (*Biozzi* et al., 1957; *Howard* et al., 1959; *Mackaness* and *Blanden*, 1967).

The results of several experiments on the resistance of H and L mice against Salm. tm. infection are summarized in Table 17 (*Biozzi*, 1972). As expected the antibody response to both flagellar and somatic antigen of Salm. tm. was much stronger in H than in L mice (Fig. 17). On the contrary, the natural resistance to this pathogen was stronger in L than in H mice. This difference was detectable in terms of mean survival time when an extremely severe infection was produced by intraperitoneal challenge. When the severity of the infection was decreased by subcutaneous challenge, the stronger resistance of L line was evident in terms of definitive survival. Both low virulence Salm. tm. vaccination and BCG pretreatment allowed a 90%–100% survival of L mice against a 100% lethal infection in controls. No survival was observed in pretreated

Table 17. Natural resistance and protective effect of BCG and of vaccination against Salm. tm. infection in H and L lines

	Challenge Number of Salm. tm. injected	H line		L line	
		per cent mortality	Mean survival time (days)	per cent mortality	Mean survival time (days)
Control	1000 I.P.	100	5.4	100	8.7
Natural resistance	5000 S.C.	100	10.4	45	—
Specific vaccination with living low virulence Salm. tm.	1000 I.P.	100	8.6	10	—
Non-specific protection[a] with living M. tuberculosis BCG strain 14 days previously	1000 I.P.	100	11.9	0	—

From *Dodin* et al., 1972.
From *Biozzi* et al., 1972c.
[a] BCG 4×10^6 viable units i.v.

H mice which showed only an increase of the mean survival time compared to the controls. Thus both natural and induced resistance to Salm. tm. infection were stronger in L antibody responders endowed with very active macrophages.

Identical results were obtained in the severe infection induced by subcutaneous inoculation of 1000 highly virulent Yersinia pestis. This severe challenge produced a 100% mortality in both lines. The stronger natural resistance of L mice was demonstrated by a mean survival time of 7.7 days compared to that of H mice (4.5 days). This interline difference was markedly amplified after vaccination with 500 μg of protective Y. pestis extract. No protection was produced in H mice which all died within a mean survival time of 5 days, while 100% of the vaccinated L mice survived the infection (*Dodin* et al., 1972).

A study of Brucella suis (B. suis) infection in H and L mice was carried out by *Cannat* et al. (1978). The antibody response to B. suis was 16-fold higher in H than in L mice. As predictable by the results of Chapter V, the delayed cutaneous hypersensitivity to melitin was of similar intensity in the two lines. Both natural and post-vaccinal resistances to B. suis infection were stronger in L than in H mice. In these experiments the resistance to infection was measured by the number of surviving B. suis inside spleen macrophages. The bactericidal activity of macrophages constitutes the principal defence against B. suis infection while antibodies only play an accessory role.

Howard (personal communication) studied, in H and L mice of Selection I, the natural resistance to Leishmania tropica (L. tropica), a typical parasite of macrophages. Although the antibody response to L. tropica antigens was much higher in H than in L line, the resistance against this infection was stronger in L line. The subcutaneous injection of this parasite produced in L mice small and transient local skin lesions which healed within 1–2 months

and all the mice survived. In contrast, in H mice the local lesions were extremely large, causing a high percentage of mortality. The comparison of L. tropica survival inside the peritoneal macrophages of H and L mice confirmed the primordial importance of these cells for the natural resistance against this infection.

All the above-mentioned examples demonstrate the higher resistance of L mice against "intracellular parasite" infections.

2. Antibody Dependent Immunity

An antibody dependent host defence mechanism operates in Trypanosoma and Plasmodia infections.

Trypanosoma cruzi (T. cruzi) infection was studied in H and L mice by *Kierszenbaum* and *Howard* (1976). The antibody titre to T. cruzi antigens, after injection of a sublethal dose of parasite was 1/945 in H mice and $<1/20$ in L mice. The natural resistance to this infection was markedly higher in H than in L mice. The dose of living parasites inducing a 50% mortality (LD 50) was 270–1 000 times larger in H than in L mice.

An active specific vaccination failed to protect L mice while an efficient protection was produced in H mice. It was clearly demonstrated that the inefficacy of vaccination in L mice was due to their genetic defects in antibody response since protection could be induced in these mice by passive administration of immune serum from vaccinated H mice.

Antibody dependent post-vaccinal immunity was also demonstrated in Plasmodium berghei (P. berghei) infection as shown in Table 18.

The similar percentage of mortality and survival time in H and L infected controls indicate that the natural resistance against P. berghei infection is independent from both antibody response and macrophage activity.

The protective effect of vaccination, on the contrary, is much stronger in H than in L mice. The antibody titre to P. berghei measured by immunofluorescence in vaccinated mice was 1/11 000 in H mice and 1/1 000 in L mice. Similar results have been obtained in H and L mice of Selection II. The concordance

Table 18. Natural resistance and protective effect of specific vaccination in Plasmodium Berghei infection

	H line		L line	
	Mortality	Mean survival time (days)	Mortality	Mean survival time (days)
Control mice	84%	16.6	95%	17.7
Vaccinated mice	5%	—	85%	19

Biozzi et al., 1978.
Infection: 10^7 i.p. parasitized mouse erythrocytes.
Vaccination: 6 i.p. injections a week apart from 3×10^7 parasitized mouse erythrocytes irradiated with 60 000 r.
(This investigation received financial support from the World Health Organization)

of results in Selections I and II substanciates the importance of antibody response in the mechanism of post vaccinal immunity. Moreover, in Selection II, a positive correlation ($r=0.88$) was found between the antibody titre in the vaccinated mice and the percentage of survival to P. berghei infection in H and L lines and their interline hybrids F_1, BcH and BcL.

Recently, *Nilsson* et al. (in press) investigated the antibody response to rabies virus and the protective effect of vaccination in H and L mice of Selections III and IV. The antibody titre measured by virus neutralization test and the resistance to infection induced by intracerebral challenge were determined. In Selection III the antibody titre was about 10-fold higher in H than in L mice; this difference was smaller in Selection IV. The natural resistance of non-vaccinated mice was similar in H and L lines of both Selections. The protective effect of vaccination was stronger in the H lines than in the L lines of the two Selections. This effect was more pronounced in Selection III than in Selection IV. The efficacy of vaccination was roughly related to the level of antibody response of the four lines of mice.

B. Anti-Helminthic Immunity

Immunity against large multicellular parasites such as Schistosoma mansoni (S. mansoni) (*Blum* and *Cioli*, 1978), and Trichinella spiralis (T. spiralis) (*Perrudet-Badoux* et al., 1975, 1978) was investigated in H and L lines of Selection I. The antibody response to antigens extracted from these two parasites was always markedly higher in H mice.

The innate resistance to S. mansoni infection was stronger in L than in H mice, moreover L mice developed an acquired resistance to re-infection as good and even better than that of H mice. In this infection therefore, antibodies do not seem to play the efficient protective role which is rather to be attributed to macrophages (*Blum* and *Cioli*, 1978).

The IgG_1 and IgE antibody titre to T. spiralis antigens was 25- to 50-fold higher in H than in L mice (*Perrudet-Badoux* et al., 1975). The level of natural resistance to primary infection produced by a low number of larvae (50) was higher in H line, nevertheless L mice acquired a total protection against re-infection while only a partial protection was acquired by H mice. These results suggest that immunity to primary or secondary T. spiralis infection depends on different mechanisms (*Perrudet-Badoux* et al., 1978).

C. Anti-Tumoral Immunity

1. Allogeneic Tumors

Two allogeneic tumors were transplanted in H and L mice: the Ehrlich ascitic carcinoma and the Sarcoma 180.

The susceptibility to Ehrlich carcinoma was similar in H and L lines: a 100% mortality and an equivalent survival time was observed in both lines.

In contrast, a striking superiority of L line resistance against Sarcoma 180 was demonstrated. This tumor grew rapidly in H line, inducing a 90% mortality within 50 days. In L line, on the contrary, after an initial growth the tumor regressed and disappeared in all the mice. This striking difference between the results obtained in Ehrlich carcinoma and in Sarcoma 180 is very probably due to the high sensitivity of Sarcoma 180 to the enhancing effect of antibody whilst the Ehrlich tumor is resistant to enhancement (*Biozzi* et al., 1972c). Some unpublished experiments of transfer to L mice, of serum from H mice bearing tumors, suggest that the growth of Sarcoma 180 in L line was suppressed by lack of enhancing antibody response. The possible contribution of the active macrophages of L mice in tumor immunity is not excluded.

2. Syngeneic Tumors

The syngeneic DBA/2 and AKR leukemia, the C_3H mammary carcinoma and strain XVII lymphosarcoma were studied. These tumors only grow in the syngeneic strain, not in H nor L mice. Therefore the study was carried out in F_1 hybrids between the syngeneic strains and H or L mice. It was previously demonstrated that these F_1 hybrids retain a part of the parental interline difference in antibody responsiveness characterizing H and L lines. The tumor induced mortality and the rate of tumor growth was similar in H and L F_1 hybrids (*Biozzi* et al., 1972c). According to the model presented in Table 16, if immune resistance were induced during the growth of these tumors, it should be due to cell mediated immunity without decisive participation of either macrophage activity or antibody response.

3. Carcinogen Induced Tumors

The tumors were induced in mice of Selection I and Selection II by intramuscular injection of 200 μg of 3–4 benzopyrene in oil solution. In both Selections the tumor incidence was higher in L than in H line. Nine months after carcinogen injection the tumor incidence was 13% in H line and 52% in L line of Selection I (*Biozzi* et al., 1972c). In Selection II the tumor incidence was 35% in H line and 54% in L line. The interline difference in tumor incidence was larger in Selection I than in Selection II, this may be due to the difference in the non-specific effect of the two Selections on responsiveness to unrelated antigens which is smaller in Selection II than in Selection I (Chapter IV).

The incidence of 3–4 benzopyrene induced tumors was investigated in interline hybrids: F_1, BcH and BcL of Selection I, and compared with the antibody response to a threshold dose of EA. This antigen was chosen since the supposed tumor antigens are unrelated to the selection antigens. The choice of the threshold dose is justified since it is generally admitted that tumor antigens are present in minute amounts in the initial stage of tumor development when anti-tumor immune defence is likely to be effective.

The results of this experiment, reported in Fig. 22, show a high correlation ($r = 0.91$) between the antibody response and the anti-tumor resistance measured

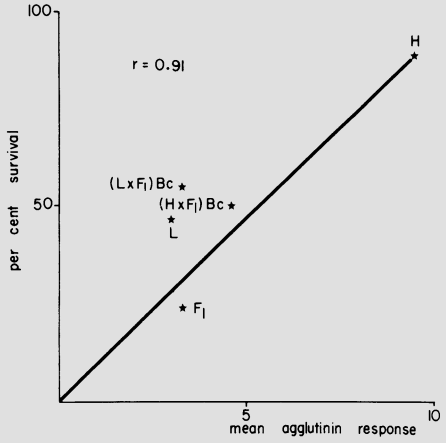

Fig. 22. Correlation between resistance to carcinogen induced tumours and responsiveness to threshold dose of unrelated antigen in H and L lines of Selection I and their interline hybrids. Per cent survival in groups of 30–36 mice 9 months after intra-muscular injection of 200 μg 3.4 benzopyrene plotted against the mean of maximal agglutinin titres after i.p. injection of 0.05 μg EA in groups of 10–20 mice (correlation calculated by a least square regression curve)

by the survival percentage. These findings do not demonstrate but suggest a possible role of antibody responsiveness in the resistance to carcinogen induced tumors. In this respect it should be remembered that antibody responsiveness to the threshold dose of EA is a recessive character in F_1 hybrids and is controlled by a single H-2 linked locus (Chapter IV.A.). The importance of H-2 linked genes in the anti-tumor immunity is an open possibility while their intervention in anti-infection immunity is unlikely, as hypothesized in the introduction.

4. Incidence of Spontaneous Tumors

This study was performed by *Covelli* et al. (1978) in H and L mice of Selection I. The mice were kept in the same animal department until spontaneous death. They were then submitted to systemic macroscopic and histologic examination.

The first finding was that L mice had a shorter life expectancy than H mice. The mean life span was about 450 days in L line and 700 days in H line. This interline difference in longevity was precedently observed by Howard in colonies of H and L mice bred in his animal department (personal communication).

The observations of neoplastic pathology by *Covelli* et al. demonstrated that the incidence of malignant tumors was 4% in H mice and 32% in L mice ($p < 0.025$). The majority of tumors were generalized lymphomas and lung invasive adenocarcinomas sometimes associated with metastasis. The occurrence of lymphomas was about 10-fold more frequent in L than in H mice ($p < 0.05$). This interline difference in spontaneous tumor incidence is large enough to have a significant impact on longevity.

This study, like the preceding one on the carcinogen induced tumors, does not demonstrate, but clearly suggests a possible causal relation between antibody responsiveness and anti-tumor immunity.

VII. Summary

The study of the genetic regulation of immune response to natural multideterminant immunogens was undertaken by the method of bidirectional selective breeding of High or Low antibody responder lines of mice. Five Selections are described:

Selection I, carried out for agglutinin responsiveness to sheep erythrocytes and pigeon erythrocytes alternated in each generation.

Selection II, carried out for agglutinin responsiveness to sheep erythrocytes repeated in each generation.

Selection III and Selection IV performed respectively for agglutinin response to flagellar or somatic antigens of Salmonella typhimurium and Salmonella oranienburg alternated in each generation.

Selection V, performed for passive agglutinin response to bovine serum albumin and rabbit gamma globulin alternated in each generation.

In each Selection the character investigated is polygenic. High and Low responder lines diverge progressively during the selective breeding. The maximal interline separation (selection limit) is reached in the 7th–16th generations. High and Low responder lines at selection limit are considered homozygous for the character submitted to selection. Their variance is therefore only due to environmental effects.

The difference in agglutinin titre between High and Low lines is 220-fold in Selection I, 103-fold in Selection II, 90-fold in Selection III, 85-fold in Selection IV and 275-fold in Selection V.

The partition of genetic and environmental variances in the foundation populations of the five Selections is established. The proportion of genetic variance is 60% in Selection I; 49% in Selection II; 51% in Selection III; 47% in Selection IV and 76% in Selection V.

The heritability of the character investigated is about 0.20 in the five Selections.

The approximate number of independent loci regulating the quantitative antibody response is between 9–15 in Selection I, 2–12 in Selection II, 7–18 in Selection III, 5–12 in Selection IV and 2–4 in Selection V.

The effect of the polygenic regulation of responsiveness to selection antigens is essentially non-specific. The same group of genes regulates the antibody response to many complex immunogens unrelated with those used during the selective breeding. The extent of this non-specific effect depends on the nature of the antigens and on the immunization procedure. It is very large in Selections I and III, intermediate in Selections II and IV, and restricted in Selection V.

The immunobiologic effect of the genes regulating antibody responsiveness was extensively studied in Selection I. The principal results are:

1. The selective breeding only affects antibody responsiveness. The cell mediated immune responses have the same intensity in High and Low antibody responder lines.

2. The metabolic and bactericidal activities of macrophages are markedly higher in Low than in High antibody responder line.

The natural resistance against various experimental infections and tumors, and the protective efficiency of vaccination were studied. The genetic independence of the immunity functions permits the identification of the limiting factor responsible for the anti-infection immunity.

Acknowledgment. The excellent secretarial assistance of Simone Mandiola in the preparation of the manuscript was greately appreciated.

References

André, C., Bazin, H., Biozzi, G.: IgA antibody response to intragastric immunization in high and low immune responder lines of mice. Eur. J. Immunol. *7*, 246–248 (1977)

Baker, P.J., Amsbaugh, D.F., Prescott, B., Stashak, P.W.: Genetic control of the antibody response to type III pneumococcal polysaccharide in mice. III. Analysis of genes governing the expression of regulatory T cell activity. J. Immunogenet. *3*, 275–286 (1976)

Benacerraf, B.: The genetic control of specific immune responses. Harvey lectures. New York: Academic Press 1973

Benacerraf, B., Dorf, M.E.: Genetic control of specific immune responses. In: Progress in immunol. II. *Brent, L., Holborow, J.* (eds.), Vol. II, pp. 181–190. Amsterdam: North-Holland 1974

Benacerraf, B., McDevitt, H.O.: Histocompatibility-linked immune response genes. Science *175*, 273–279 (1972)

Biozzi, G.: Differential susceptibility of high and low antibody producing mouse lines to infection and tumor transplantation. In: Genetic control of immuneresponsiveness. *McDevitt, H.O., Landy, M.* (eds.), pp. 317–322. New York: Academic Press 1972

Biozzi, G., Halpern, B., Benacerraf, B., Stiffel, C.: Phagocytic activity of the RES in experimental infections. In: Physiopathology of the RES. *Halpern, B., Benacerraf, B., Delafresnaye, J.F.* (eds.), pp. 204–225. Oxford: Blackwell 1957

Biozzi, G., Asofsky, R., Lieberman, R., Stiffel, C., Mouton, D., Benacerraf, B.: Serum concentrations and allotypes of immunoglobulins in two lines of mice genetically selected for "high" and "low" antibody synthesis. J. Exp. Med. *132*, 752–764 (1970)

Biozzi, G., Stiffel, C., Mouton, D., Bouthillier, Y., Decreusefond, C.: Genetic regulation of the function of antibody producing cells. In: Progress in immunol. *Amos, B.* (ed.), pp. 529–545. New York: Academic Press 1971

Biozzi, G., Stiffel, C., Mouton, D., Bouthillier, Y., Decreusefond, C.: Cytodynamics of the immune response in two lines of mice genetically selected for "high" and "low" antibody synthesis. J. Exp. Med. *135*, 1071–1094 (1972a)

Biozzi, G., Stiffel, C., Mouton, D., Bouthillier, Y., Decreusefond, C.: Morphology and differentiation of rosette forming cells during the immune response. Transplant. Proc. *4*, 339–343 (1972b)

Biozzi, G., Stiffel, C., Mouton, D., Bouthillier, Y., Decreusefond, C.: Importance de l'immunité spécifique et non spécifique dans la défense antitumorale. Ann. Inst. Pasteur Paris *122*, 685–694 (1972c)

Biozzi, G., Stiffel, C., Mouton, D., Bouthillier, Y., Decreusefond, C.: La régulation génétique de la synthèse des immunoglobulines au cours de la reponse immunologique. Ann. Immunol. (Paris) *125C* 107–142 (1974)

Biozzi, G., Stiffel, C., Mouton, D., Bouthillier, Y.: Selection of lines of mice with high and low antibody responses to complex immunogens. In: Immunogenetics and immunodeficiency. *Benacerraf, B.* (ed.), pp. 179–227. Lancaster: MTP 1975

Biozzi, G., Mouton, D., Heumann, A.M., Bouthillier, Y., Stiffel, C., Mevel, J.C.: Genetic analysis of antibody responsiveness to sheep erythrocytes in crosses between lines of mice selected for high or low antibody synthesis. Immunology *36*, 437–438 (1979)

Biozzi, G., Mouton, D., Stiffel, C., Sant'Anna, O.A., Bouthillier, Y.: The genetic regulation of antibody responsiveness to natural immunogens in relation to the protective effect

of vaccination. In: Pharmacology of immunoregulation. *Werner, G.H., Floc'h, F.* (eds.), pp. 123–139. New York: Academic Press 1978

Blum, K., Cioli, D.: Paradoxical behaviour of Biozzi high and low responder mice upon infection with Schistosoma mansoni. Eur. J. Immunol. *8*, 52–56 (1978)

Bodmer, W.F., Cavalli-Sforza, L.L.: Genetic, evolution and man. San Francisco: Freeman 1976

Byfield, P.E., Howard, J.G.: Equivalent graft versus host reactivity of spleen cells from two lines of mice genetically selected for high and low humoral antibody formation. Transplantation *14*, 133–134 (1972)

Cannat, A., Bousquet, C., Serre, A.: Response of High and Low antibody producers to Brucella. Ann. Immunol. (Paris) *129c*, 669–683 (1978)

Cavalli-Sforza, L.L., Bodmer, W.F.: The genetics of human populations. San Francisco: Freeman 1971

Chase, M.W.: Inheritance in guinea pigs of the susceptibility to skin sensitization with simple chemical compounds. J. Exp. Med. *73*, 711–726 (1941)

Covelli, V., Di Majo, V., Bassani, B., Metalli, P.: Life span and spontaneous tumours in mice selected for high and low antibody responses (Biozzi mice). Br. J. Cancer *38*, 472–475 (1978)

Del Guercio, P., Zola, H.: A comparison of the immune response to DNP in mice genetically selected for High or Low antibody synthesis. Immunochemistry *9*, 769 (1972)

Dodin, A., Wiart, J., Stiffel, C., Bouthillier, Y., Mouton, D., Decreusefond, C., Biozzi, G.: Résistance à l'infection par Salmonella typhimurium chez les souris des lignées génétiquement sélectionnées d'après le caractère «production d'anticorps». Ann. Inst. Pasteur Paris *123*, 137 (1972)

Doria, G., Agarossi, G., Biozzi, G.: In vitro immune response of spleen cells from mice genetically selected for high and low antibody production. Immunology *34*, 999–1005 (1978)

Fachet, J., Ando, I.: Inverse regulation of cell mediated and humoral immune response to oxazolone by H-2 linked genes. Nature *273*, 239 (1978)

Falconer, D.S.: Introduction to quantitative genetics. New York: Ronald Press 1960

Feingold, N., Feingold, J., Mouton, D., Bouthillier, Y., Stiffel, C., Biozzi, G.: Polygenic regulation of antibody synthesis to sheep erythrocytes in the mouse: a genetic analysis. Eur. J. Immunol. *6*, 43–51 (1976)

Floc'h, F., Werner, G.H.: Comparative studies on susceptibility to some virus infection of mice selected with respect to their antibody responsiveness to an unrelated antigen. In: Pharmacology of immunoregulation. *Werner, G.H., Floc'h, F.* (eds.), pp. 153–161. New York: Academic Press 1978

Heumann, A.M., Stiffel, C.: Réponse immunologique des souris de lignées bonnes et mauvaises productrices d'anticorps envers un antigène non apparenté à l'antigène de sélection. Ann. Immunol. (Paris) *129C*, 13–27 (1978)

Howard, J.G., Biozzi, G., Halpern, B.N., Stiffel, C., Mouton, D.: The effect of Mycobacterium Tuberculosis (BCG) infection on the resistance of mice to bacterial endotoxin and Salmonella Enteritidis infection. Br. J. Exp. Pathol. *40*, 281–290 (1959)

Howard, J.G., Christie, G.H., Courtenay, B.M., Biozzi, G.: Studies on immunological paralysis. VIII. Pneumococcal polysaccharide tolerance and immunity differences between the Biozzi high and low responder lines of mice. Eur. J. Immunol. *2*, 269–273 (1972)

Howard, J.G., Courtenay, B.M., Desaymard, C.: Equivalent responsiveness to branched polysaccharides and their dinitrophenyl conjugates in the Biozzi high and low responder lines of mice. Eur. J. Immunol. *4*, 453–457 (1974)

Ishizaka, S., Otani, S., Morisawa, S.: Effects of carrageenan on immune responses. II. A possible regulatory role of macrophages in the immune responses of low responder mice. J. Immunol. *120*, 61–65 (1978)

Katz, D., Benacerraf, B.: Genetic control of lymphocytes interaction and differentiation. In: The role of products of the histocompatibility gene complex in immune response. *Katz, D., Benacerraf, B.* (eds.), pp. 355–385. New York: Academic Press 1976

Kierszenbaum, F., Howard, J.G.: Mechanisms of resistance against experimental trypanosoma cruzi infection: the importance of antibodies and antibody-forming capacity in the Biozzi High and Low responder mice. J. Immunol. *116*, 1208–1211 (1976)

Klein, J.: Genetic control of virus susceptibility. In: Biology of the mouse histocompatibility 2 complex. p. 385. Berlin: Springer 1975

Liacopoulos-Briot, M., Bouthillier, Y., Mouton, D., Lambert, F., Decreusefond, C., Stiffel, C., Biozzi, G.: Comparison of skin allograft rejection and cytotoxic antibody production in two lines of mice genetically selected for "high" and "low" antibody synthesis. Transplantation *14*, 590–596 (1972)

Liacopoulos-Briot, M., Mouton, D., Stiffel, C., Lambert, F., Decreusefond, C., Bouthillier, Y., Biozzi, G.: Réponse à la phytohémagglutinine des lymphocytes de 2 lignées de souris génétiquement sélectionnées pour une forte et une faible synthèse d'anticorps. Ann. Immunol. (Paris) *125C*, 393–404 (1974)

Lieberman, R., Stiffel, C., Asofsky, R., Mouton, D., Biozzi, G., Benacerraf, B.: Genetic factors controlling anti sheep erythrocyte antibody response and immunoglobulin synthesis in backcross and F_2 progeny of mice genetically selected for "high" or "low" antibody synthesis. J. Exp. Med. *136*, 790–798 (1972)

Lubet, M.T., Kettman, J.R.: Primary antibody and delayed type hypersensitivity response of mice to ovalbumin. Immunogenetics *6*, 69–79 (1978)

Mackaness, G.B., Blanden, R.V.: Cellular immunity. Prog. Allergy *11*, 89–140 (1967)

McDevitt, H.O., Benacerraf, B.: Genetic control of specific immune responses. Adv. Immunol. *11*, 31–74 (1969)

McDevitt, H.O., Oldstone, M.B.A., Pincus, T.: Histocompatibility-linked genetic control of specific immune response to viral antigen. Transplant. Rev. *19*, 209 (1974)

Meruelo, D., Lieberman, M., Deak, B., McDevitt, H.O.: Genetic control of radiation leukemia virus induced tumorigenesis. J. Exp. Med. *146*, 1088–1095 (1977)

Miller, J.F.A.P., Vadas, M.A., Whitelaw, A., Gamble, J.: H-2 linked Ir gene regulation of delayed-type hypersensitivity. In: The role of products of the histocompatibility gene complex in immune responses. *Katz, D., Benacerraf, B.* (eds.), pp. 403–415. New York: Academic Press 1976

Mishell, R.I., Dutton, R.W.: Immunization of dissociated spleen cells cultures from normal mice. J. Exp. Med. *126*, 423 (1967)

Mosier, D.E.: A requirement for two cell types for antibody formation "in vitro". Science *158*, 1575 (1967)

Mouton, D., Bouthillier, Y., Oriol, R., Decreusefond, C., Stiffel, C., Biozzi, G.: Intensité de la réaction d'hypersensibilité retardée chez les souris des lignées sélectionnées «bonnes» et «mauvaises» productrices d'anticorps. Ann. Immunol. (Paris) *125C*, 581–588 (1974)

Mouton, D. Bouthillier, Y., Heumann, A.M., Stiffel, C., Biozzi, G.: Genetic regulation of macrophage functions. In: The reticuloendothelial system in health and disease: functions and characteristics. *Reichard, S.M., Escobar, M.R., Friedman, H.* (eds.), pp. 225–236. New York: Plenum 1976

Mouton, D., Bouthillier, Y., Mevel, J.C., Prouvost-Danon, A., Biozzi, G.: Participation du locus H-2 à la détermination de la capacité de réponse à des doses faibles d'antigène chez les souris sélectionnées bonnes et mauvaises productrices d'anticorps (BP, MP). Ann. Immunol. Paris *128C*, 20–21 (1977)

Mouton, D., Heumann, A.M., Bouthillier, Y., Mevel, J.C., Biozzi, G.: Interaction of H-2 and non H-2 linked genes in the regulation of antibody response to a threshold dose of sheep erythrocytes. To be published

Mozes, E., Shearer, G.M.: Genetic control of immune response. Curr. Top. Microbiol. Immunol. *59*, 167–200 (1972)

Nilsson, M.R., Sant'Anna, O.A., Siqueira, M., Nilsson, T.T., Gennari, M.: Rabies virus immunity in genetically selected high and low responder lines of mice. Infect. Immun. (in press)

Oriol, R., Stiffel, C., Mouton, D., Bouthillier, Y., Decreusefond, C., Biozzi, G.: Catabolisme des anticorps chez les souris des lignées génétiquement sélectionnées d'après le caractère «production d'anticorps». Ann. Inst. Pasteur Paris *123*, 137 (1972)

Passos, H.C., Siqueira, M., Reis, M.H., Ferreira, V.C.A., Ibanez, O.M., Sant'Anna, O.A., Biozzi, G.: Genetic control of immune response to protein antigens. I. Two-way selective breeding of mice for quantitative antibody responsiveness to bovine serum albumin and rabbit gamma globulin. J. Immunol. *119*, 1439–1443 (1977)

Perrudet-Badoux, A., Binaghi, R.A., Biozzi, G.: Trichinella infestation in mice genetically selected for High and Low antibody production. Immunology *29*, 387–390 (1975)

Perrudet-Badoux, A., Binaghi, R.A., Boussac-Aron, Y.: Trichinella spiralis infection in mice: mechanism of the resistance in animals genetically selected for high and low antibody production. Immunology *35*, 519–522 (1978)

Prouvost-Danon, A., Stiffel, C., Mouton, D., Biozzi, G.: Anaphylactic antibodies in mice genetically selected for antibody production. Immunology *20*, 25–27 (1971)

Prouvost-Danon, A., Mouton, D., Abadie, A., Mevel, J.C., Biozzi, G.: Genetic regulation of IgE and agglutinating antibody synthesis in lines of mice selected for high and low immune responsiveness. Eur. J. Immunol. *6*, 342–348 (1977)

Rosenthal, A.L.: Determinant selection and macrophage function in genetic control of the immune response. Immunol. Rev. *40*, 136–152 (1978)

Sant'Anna, O.A., Bouthillier, Y., Siqueira, M., Biozzi, G.: Differences in the genetic control of primary and secondary antibody responses. Immunology 1979 (in press)

Sela, M.: Antigen design and immune response. Harvey lectures. New York: Academic Press 1972

Shultz, L.D., Bailey, D.W.: Genetic control of contact sensitivity in mice. Effect of H-2 and non H-2 loci. Immunogenetics *1*, 570–583 (1975)

Siqueira, M., Bandieri, A., Reis, M.H., Sant'Anna, O.A., Biozzi, G.: Selective breeding of mice for antibody responsiveness to flagellar and somatic antigens of Salmonellae. Eur. J. Immunol. *6*, 241–249 (1976)

Siqueira, M., Esteves, M.B., Ibanez, O.M., Ferreira, V.C.A., Sant'Anna, O.A., Reis, M.H., Biozzi, G.: Non specific regulation of antibody responsiveness in the mouse. Eur. J. Immunol. *7*, 195–203 (1977)

Stiffel, C., Mouton, D., Bouthillier, Y., Heumann, A.M., Decreusefond, C., Mevel, J.C., Biozzi, G.: Polygenic regulation of general antibody synthesis in the mouse. In: Progress in immunology II, vol. II, Biological aspects I. *Brent, L., Holborow, J.* (eds.), pp. 203–212. Amsterdam: North-Holland 1974

Unanue, E.R., Biozzi, G., Benacerraf, B.: Immunocytochemical studies on B lymphocytes from the Biozzi High and Low lines of mice selected for their responses to heterologous erythrocytes. Proc. Soc. Exp. Biol. Med. *145*, 1243–1249 (1974)

Vaz, N.M., Vaz, E.M., Levine, B.B.: Relationship between histocompatibility (H-2) genotype and immune responsiveness to low doses of ovalbumin in the mouse. J. Immunol. *104*, 1572–1574 (1970)

Vaz, N.M., Phillips-Quagliata, J.M., Levine, B.B., Vaz, E.M.: H-2 linked control of immune responsiveness of mice to ovalbumin and ovomucoid. J. Exp. Med. *134*, 1335–1348 (1971)

Wiener, E., Bandieri, A.: Differences in antigen handling by peritoneal macrophages from the Biozzi high and low responder lines of mice. Eur. J. Immunol. *4*, 457–463 (1974)

Zinkernagel, R.M., Doherty, P.C.: H-2 compatibility requirement for T-cell mediated lysis of target cells infected with lymphocytic choriomeningitis virus. J. Exp. Med. *141*, 1427–1436 (1975)

Chemistry and Biology of the Enterobacterial Common Antigen (ECA)

HUBERT MAYER [1] and GÜNTER SCHMIDT [1]

I. Introduction

The classical surface antigens of Enterobacteriaceae: O, K, and H antigens have been studied extensively in the past and have provided a basis for the taxonomy of this family (*Kauffmann,* 1966, 1975).

In comparison to the vast knowledge accumulated on the structure, localization, genetic determination, and biologic effects of O and K antigens (*Weinbaum* et al., 1971; *Ørskov* et al., 1977), the precise knowledge of antigens shared by various microorganisms is small. This is most probably due to the fact that the "... direction of research in the past has to a great deal been stimulated by problems and questions of taxonomy, etiology of diseases and epidemiology" (*Neter* and *Whang,* 1972).

[1] Max-Planck-Institut für Immunbiologie, Stübeweg 51, D-7800 Freiburg i.Br., Federal Republic of Germany

Only in recent years have antigens common to many species or pathogens received greater attention, especially from the point of view of their potential diagnostic and prophylactic possibilities (*Chedid* et al., 1968; *McCabe,* 1972). This might be the reason why an antigen common to all Enterobacteriaceae and designated as enterobacterial common antigen (CA or ECA) was not discovered until 1962 (*Kunin* et al., 1962). After the discovery of the "Kunin-antigen," however, much research was devoted to the elucidation of its chemical structure, its biologic properties, and its distribution among bacterial families.

Several reviews on ECA have been published (*Neter* and *Whang*, 1972; *Domingue* and *Johnson* 1975; *Gorzynski*, 1976; *Mäkelä* and *Mayer*, 1976). Since the chemical identity and important discoveries on its genetic determination have only been resolved during the last 2 years, a new survey on ECA seems justified. It is the aim of this review to cover the whole field of ECA research, but greater emphasis is placed on more recent work not included in earlier reviews. It will be obvious from this review that much more work has to be done for a thorough understanding of the chemical and biologic properties of this antigen, and especially for evaluation of its possible importance for diagnosis and prophylaxis.

II. Discovery and Modes of Detection

Enterobacterial common antigen was detected by Kunin (*Kunin* et al., 1962) when screening the antigenic cross relations between the almost 150 *Escherichia coli* O groups then known by means of the passive hemagglutination test. The latter technique, introduced by *Neter* et al. (1952) and *Neter* (1965) for the study of enterobacterial O antigens, was chosen by Kunin because of its high sensitivity and easy performance. Cross-reactions between O-test strains were much less frequent than those reported by bacterial agglutination (*Kunin* and *Beard,* 1963). Another observation, however, was most striking, namely the capacity of certain rabbit antisera to hemagglutinate cells coated with O-antigen preparations from many different Enterobacteriaceae. Thus, supernatants of heated suspensions of *Proteus, Shigella, Salmonella, E. coli, Erwinea carotovora, Klebsiella,* and even of some rough mutants of *E. coli*, isolated from clinical material, could be used for erythrocyte coating.

The heterogenetic serological activity was most prominent in rabbit antisera against *E. coli* 014 [1] (obtained from various sources), but antisera against *E. coli* 056, 0124, and 0144 were also active, although to a much lesser extent (*Kunin* et al., 1962; *Kunin* and *Beard,* 1963; *Johns* et al., 1973).

For coating erythrocytes (human or sheep) the bacterial growth of one Petri dish is harvested in 10 ml saline, boiled for 2 h at 100° C, then centrifuged at 5000 × g (15 min, 0° C). After addition of 1 ml 95% aqueous ethanol to the supernate, aliquots of a 1:10 dilution of the crude antigen preparation are incubated at 37° C for 1 h in a water-bath shaker with a 2.5% suspension of erythrocytes. After incubation, the sensitized erythrocytes are washed three

[1] As discussed on p. 126, E. coli 014:K 7 is not an S form, but an encapsulated R mutant of the R 4 core type.

times with saline or phosphate buffered saline, then readjusted to a final 2.5% concentration with buffer and used directly for hemagglutination in disposable plastic microtiter trays.

This technique is used by several groups and with several modifications. It can also be used in the hemolytic modification (see below). Passive hemagglutination and hemolysis can be inhibited by ECA, and this assay has been of considerable value for semiquantitative determinations of ECA (*Kunin,* 1963; *Suzuki* et al., 1964b).

The cross-reactive fraction of antibodies could be removed from an *E. coli* 014 antiserum by absorption with erythrocytes coated with the crude antigen extract of any *E. coli* or *Salmonella* strain. The 014-specific fraction was however, completely retained (*Kunin* et al., 1962). These results proved that 014 antiserum contained antibodies with two non related specificities: one to an antigen common to all tested enterobacterial strains and the other specific for the 014 lipopolysaccharide (LPS).

It was surprising that other enterobacterial antisera did not contain detectable amounts of antibodies to ECA, although all bacteria contained this antigen. It was first thought that endotoxin preparations contain two antigenic specifities (antigenic determinants): one the classical specific O antigen, the other a hapten common to almost all members of the Enterobacteriaceae family (*Kunin* et al., 1962). In the 014 strain the common hapten appeared to be situated or structured in such a way that it can behave as complete antigen and induce antibody formation.

Immediately after the discovery of ECA, *Whang* and *Neter* (1962) confirmed the findings; they showed in addition that, although crude preparations of endotoxin contained ECA, purified LPS was devoid of it. This indicates that O and common antigen are not necessarily associated. They showed further that supernate fluids of unheated agar-grown suspensions contained only small amounts of ECA, but that boiling of the bacterial suspension releases additional and substantial quantities of it. For quantitation of ECA they used the passive hemagglutination inhibition test and for demonstration of ECA antibodies, the passive hemolysis test, a hemolytic modification of the passive hemagglutination test. Passive hemolysis is slightly more sensitive than the hemagglutination method (*Whang* and *Neter,* 1962).

In the hemolysis test, guinea pig complement (0.1 ml of a 1:10 dilution) is added to the mixtures of antigen-coated erythrocytes and antiserum. The mixtures are incubated (37° C, 1 h) and the resulting hemolysis is read grossly. Sheep red blood cells (SRBC) have to be used, since human erythrocytes are less easily lysed in this test (*Neter,* 1956).

Hemolysis is also measured in the Jerne plaque technique developed by *Domingue* and *Neter* (1967) for demonstration of ECA antibodies produced by lymph node cells. Hemolysis is regularly observed when RBC sensitized with ECA are injected into immunized rabbits (hemoglobinuria, hemoglobinemia), but not with nonimmune animals (*Suzuki* et al., 1964b).

In later studies (*Kunin*, 1963), chromatography on DEAE (diethylaminoethyl) cellulose was found to separate ECA from O antigen, at least with some strains, and this led *Kunin* to the assumption that the ability of *E. coli* 014 to engender

ECA antibodies in experimental animals is due to the considerably higher amounts of ECA in this particular strain.

That immunogenicity of ECA, however is not a quantitative but rather a qualitative phenomenon was clearly established by *Neter* et al. (1964) when equivalent amounts of ECA from *E. coli* 014 and other enteric strains were used for immunization. Only ECA from *E. coli* 014 caused a specific immune response in the rabbit. It is now established (see p. 125ff.) that ECA exists in (at least) two modifications. The haptenic form – which only under special conditions may elicit an ECA immune response (see Sect. VII. A) – is present in all enteric bacteria, and the strains containing it are classified as "*nonimmunogenic*" strains because they do *not engender ECA antibodies* when *whole heat-killed bacterial suspensions are used for immunization* (*Whang* et al., 1972b). The "*immunogenic*" strains contain, in addition to the haptenic ECA, a fraction which is covalently linked to the R core of special genetically defined R mutants (see Sect. VII. B), and it is this fraction which accounts for the ECA immunogenicity of these strains (*Kiss* et al., 1978).

Other properties of ECA and ECA antibodies, which were at first puzzling, are now partly understood. Antibodies against ECA fail to agglutinate (heterologous) enteric bacteria containing this antigen (*Kunin* et al., 1962; *Whang* and *Neter,* 1962), although it was shown by means of fluorescent antibodies that ECA is localized on the bacterial surface (*Aoki* et al., 1966). Agglutination of ECA-modified latex particles also was not possible (*Whang* and *Neter,* 1962; 1963b), in contrast to specific O agglutination after coating with the corresponding O antigens. That latex particles were in fact coated by ECA is clearly demonstrated by the absorption of ECA antibodies by the modified particles (*Domingue* and *Neter,* 1966a) and also by the fact that modified particles are opsonized in the presence of ECA antibodies (*Domingue* and *Neter,* 1966b).

Quite recently it was shown that potent ECA antisera are able to agglutinate R mutants of *Salmonella* and *E. coli* and that the agglutination titers are of comparable height to the hemagglutination titers (*Marx* et al., 1977). The corresponding S forms are not agglutinable, indicating that ECA is probably not sufficiently exposed in S forms to allow bacterial agglutination. The specificity of the reaction was ascertained by using (absorbed) ECA-monospecific antisera.

During the initial studies on ECA and ECA antibodies, *Kunin* observed that ECA-containing fractions were not precipitated even when antisera with very high hemagglutination titers were used (*Kunin* et al., 1962; *Kunin,* 1963). This unexpected observation was soon confirmed by other authors.

Later it was found (*Mayer* and *Schmidt,* 1971) that some, but certainly not all antisera against an ECA-immunogenic R1 mutant (*E. coli* $08^-:K27^-$) were able to precipitate ECA in ECA-enriched fractions or LPS–ECA complexes as present in hot phenol–water extracts as source of antigen[2]. Phenol-chloroform-petroleum ether (PCP)-extracted LPS was not reactive. Agar gel diffusion and slide immunoelectrophoresis were used; the latter showed ECA to be strongly negatively charged and to tend to form partly superimposed precipita-

[2] It has to be mentioned here that the procedure of handling the culture, including killing, may have a striking effect on the presence or absence of ECA. For example, treatment with ethanol for killing the bacteria removed ECA from cultures used for LPS preparations.

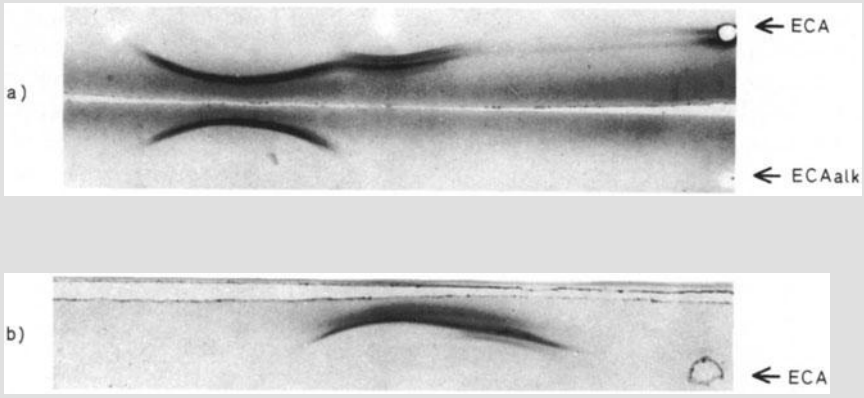

Fig. 1. Immunoelectrophoresis in agar gel of enterobacterial common antigen obtained from *Salmonella montevideo* SH94 before (ECA) and after treatment with alkali (ECAalk). Two different antisera were used, both prepared with living *Escherichia coli* 014 cells. Electrophoresis was for 1 h (a) or 45 min (b) at 10 V/cm. The agar gels were stained with Coomassie brilliant blue. *Männel* and *Mayer,* Eur. J. Biochem. *86*, 374, 1978b

tion lines (*Whang* et al., 1973; *Männel* and *Mayer,* 1978b). The characteristic precipitation pattern of highly purified ECA in immunoelectrophoresis is shown in Fig. 1, which demonstrates that ECA (at pH 8.6) is negatively charged and shows a characteristic precipitation pattern ("birdwing pattern").

Independently, *Johns* et al. (1973) used gel precipitation and immunoelectrophoresis for ECA isolation and characterization of fractions during purification. It was observed that antisera preserved in 50% glycerol were not suitable for precipitation studies because the osmotic movement of water prevented the antibodies from diffusing. Such antisera had been used in earlier studies and this might account for their failure to precipitate. A comparative analysis of aqueous extracts of ECA-containing and ECA-negative *Salmonella* mutants (*Johns* et al., 1973) documented the value of the precipitation technique for screening mutants for the presence or absence of ECA.

In a reinvestigation of the agar gel-precipitating capacities of ECA antisera (*Whang* et al., 1973), it was found that no absolute correlation exists between hemagglutinin titers and precipitating capacity. Even serum samples from a single rabbit immunized i.v. with ethanol-soluble ECA showed that precipitation occurs only with high-titered early immune sera, not with late immune sera having identical or even higher hemagglutinin titers. ECA antibodies were shown to belong predominantly to the IgM type (19S antibodies), irrespective of whether early or late antisera were studied (*Whang* et al., 1967). Hemagglutination, however, measures especially this type of antibody. A high IgG antibody response (7S antibodies) was recently obtained by *Measel* (1977) using multiple i.m. injections of rabbits. The antibodies were differentiated by a modified Coombs technique. It would be of interest to investigate the precipitating capacity of these antisera.

Following an observation by M.A. *Johns* (personal communication), rather good precipitating antisera were obtained by i.v. injection of living *E. coli* 014 suspensions (*Männel* and *Mayer*, 1978b). ECA precipitation in agar gel is now a useful method for quantitating ECA in fractions eluted from columns. Radial immunodiffusion according to *Mancini* et al. (1965) was used by *Männel* et al. (1978) and rocket immunoelectrophoresis by *Lugowski* and *Romanowska* (1978) for semiquantitative ECA determination. The observation by *Conley* et al. (1976) that ethanol extracts contain two distinct antigens that precipitate with *E. coli* 014 antiserum shows the need for using monospecific ECA antisera, which are now obtainable by immune serum absorptions with appropriate ECA-negative mutants of immunogenic strains (*Männel* and *Mayer*, 1978b).

An immunofluorescent demonstration of ECA was worked out by *Aoki* et al. (1966) with the aim of localizing enteric bacteria and released ECA in tissue specimens, especially in the case of pyelonephritis. The indirect technique was chosen with rabbit *E. coli* 014 antiserum and goat antirabbit immunoglobulin conjugated with fluorescein isothiocyanate: Bacterial smears or tissue samples on clean glass slides were air dried, fixed in acetone for 10 min, and stained by the indirect technique with 1:10 diluted 014 antiserum and undiluted fluorescein-labeled goat antiserum. Bacteria were first layered with unlabeled 014 antiserum for 1 h, and then carefully washed with PBS. Staining was accomplished with goat antirabbit antiserum using a 1-h incubation at room temperature. After washing, the stained samples were examined by fluorescent microscopy.

An excellent correlation was found between hemagglutination and the fluorescent staining in several strains of *E. coli, Klebsiella*, and *Proteus* using *Pseudomonas aeruginosa* and *Streptococcus* as negative controls. Some enteric bacteria that had small amounts of ECA or none at all when supernates were tested in passive hemagglutination were not stained. It was not further investigated whether these strains were in fact ECA-negative mutants.

E. coli 014, the homologous strain, gave the most brilliant fluorescence, and the stain was homogeneously distributed. Heterologous bacteria stained less intensely and showed irregular distribution of the fluorescence (*Aoki* et al., 1966).

Taking into consideration that a number of common antigens might be present on the bacterial surface, the use of nonabsorbed ECA antisera is not unequivocally indicative for ECA presence. In fact, it was shown that antibodies against the 014 LPS were present in much larger numbers than antibodies against ECA. In a very recent study (*Rinno*, 1979), making use of an ECA-monospecific rabbit antiserum and fluorescent antirabbit IgG, essentially the same results were obtained: brilliant and evenly distributed fluorescence with *E. coli* 014 and other ECA-immunogenic strains and a much less intense and spotty staining with ECA-containing S forms or R mutants. Genetically related ECA-negative mutant (*rfe*$^-$ mutants) were used as controls and showed no staining at all.

The possibility of staining a large number of bacterial strains with the aid of a single antiserum may be valuable for clinical studies. It can replace many specific antisera against many different bacterial strains, although the lower sensitivity might not be sufficient for all purposes (*Aoki* et al., 1967; *Thomsen* and *Hjort*, 1973; *Thomsen*, 1974).

III. Distribution in Bacteria and Role in Taxonomy

The first communication by *Kunin* et al. (1962), in which the discovery of the "common Enterobacteriaceae hapten" was reported, contains a survey on the distribution of this antigen. This hapten was demonstrable in supernates of heated bacterial cultures, but also in commercial endotoxin preparations obtained by phenol–water or trichloroacetic acid extractions of a wide variety of enterobacterial strains. Other gram-negative bacteria including *Brucella, Pseudomonas, Bordetella,* and gram-positive bacteria were found to lack this antigen. The result of this study provided the basis for *Kunin* to designate the antigen as "common Enterobacteriaceae antigen" (CA, or as it is now often abbreviated, ECA).

Following this initial observation, studies on several hundreds of gram-negative strains have been carried out, especially on species that were thought to be similar to Enterobacteriaceae (*Diaz* and *Neter,* 1969; *Whang* et al., 1972a; *Whang* and *Neter,* 1972; *Whang* and *Neter,* 1973; *Bader* 1972; *LeMinor* et al., 1972; *Maeland* and *Digranes,* 1975a). The distribution of ECA has been thoroughly reviewed by *Mäkelä* and *Mayer* (1976) and the essentials are given in Table 1.

Perusal of Table 1 shows that ECA has been detected so far only among members of Enterobacteriaceae and in *Vibrio shigelloides,* also classified as *Plesiomonas* or *Aeromonas shigelloides.* No strains of other families of gram-negative bacteria and no gram-positive bacteria so far studied produce ECA.

Table 1. Distribution of ECA in wild-type strains of gram-negative facultatively anaerobic rods

ECA is present in	ECA is lacking in
Family enterobacteriaceae	
Escherichia	
Edwardsiella	
Citrobacter (including Levinea)	
Salmonella	
Shigella	
Klebsiella	
Enterobacter	
Serratia	
Proteus	
Yersinia	
Erwinia (including Pectobacterium)	Erwinia chrysanthemi
Family vibrionaceae	
Plesiomonas shigelloides (15 strains)	
Aeromonas hydrophila 209A (1 strain)	Aeromonas
	Vibrio
Other genera of facultative anaerobic rods	Flavobacterium
	Hemophilus
	Pasteurella
	Rhodospirillaceae[a] (11 strains)
	Nitrobacteriaceae[a] (1 strain)

[a] *Mayer* unpublished.

With the exception of *Erwinia chrysanthemi*, all wild-type strains of Enterobacteriaceae produce ECA. Occasionally, however, exceptions have been reported; thus in a broad study of 213 enterobacterial strains three were found to be ECA negative (*LeMinor* et al., 1973): one of 13 *Proteus vulgaris* strains and two of 13 *Proteus mirabilis* strains. *Aoki* et al. (1966) also found three *Proteus* strains (one of each of the following species: *P. morgani, P. vulgaris, P. rettgeri*) that did not react in passive hemaggutination and did not stain when immunofluorescence was used for ECA detection.

ECA^+ strains can mutate to ECA-negative (ECA^-) strains (see Sect. V), and when this mutation concerns the *rfe* gene locus ECA^- R mutants are formed. The fact that enterobacterial R mutants are often deficient in ECA production was soon recognized (*Whang* et al., 1972b; *Johns* et al., 1973; *Mäkelä* et al., 1974), and these findings stimulated work on the genetic determination of ECA (*Mäkelä* and *Mayer*, 1974; *Schmidt* et al., 1976b). ECA was also present in glycine-induced spheroplasts of *Salmonella typhi* (*Whang* and *Neter*, 1965b) and in penicillin-induced spheroplasts of *P. mirabilis* (*Rinno*, 1978; *Männel* et al., 1977).

It is conceivable that the above-mentioned ECA^--strains of *Proteus* (*LeMinor* et al., 1973; *Aoki* et al., 1966) and the single ECA^- *E. coli* strain described by *Aoki* et al. (1966) might be mutants defective in the ECA determining *rfe/rff* genes and therefore do not represent real exceptions.

On the other hand, the lack of ECA in all six strains of *Erwinia chrysanthemi* studied (*LeMinor* et al., 1972) vs its presence in other species of *Erwinia* may reflect the heterogeneity of this genus, which is also indicated by the wide fluctuation of the GC ratio among the various species (*Starr* and *Mandel*, 1969).

The presence of ECA in *Yersinia* (*pestis, pseudotuberculosis*, and *enterocolitica*), and its absence in *Pasteurella multocida* and *haemolytica* (*LeMinor* et al., 1972; *Bader*, 1972; *Maeland* and *Digranes*, 1975a and b) is in excellent agreement with the recent change in classification, resulting in elimination of the *Yersinia* from the genus *Pasteurella* and incorporation of this new genus into the Enterobacteriaceae.

The presence of ECA in *Plesiomonas (Vibrio, Aeromonas) shigelloides* is well-established (*Whang* et al., 1972a; *LeMinor* et al., 1972), but the taxonomic position of this group of strains is not completely clear (*Buchanan* and *Gibbons*, 1974). The recently described ECA-containing strain 209A of *Aeromonas hydrophila* (*Nacescu* and *Ciufecu*, cited in *Marx* et al., 1977) surely needs a reinvestigation, because other strains of this species (9 investigated by *Whang* et al., 1972a; and 12 by *LeMinor* et al., 1972) all proved ECA negative.

These few examples may be taken to illustrate the value of ECA for taxonomic questions in cases where additional arguments or criteria are needed.

IV. Isolation Procedures and Chemical Characterization

A. Introductory Remarks

Until very recently the chemical nature of ECA remained obscure and even today some questions with regard to its chemical structure are unresolved.

The main reason for the difficulties encountered in elucidation of ECA chemistry are probably the fact that ECA is easily liberated from the bacterial cell envelope and then easily dislocated due to its high surface activity. ECA may adhere to cellular debris or macromolecules or form comicelles with other lipophilic or amphiphilic substances simultaneously present in homogenates or sonicates of bacterial cells. When isolated fractions thereafter were found to exhibit ECA activity in ECA-hemagglutinating systems, the material contaminated with ECA might have been taken erroneously as ECA. For example, phenol–water extracted lipopolysaccharide of enterobacterial wild-type strains is usually contaminated with ECA (*Kunin*, 1963), whereas PCP-extracted LPS of the same strains is not (*Mayer* and *Schmidt,* 1971).

Another difficulty encountered earlier resulted from the existence of ECA in ECA-immunogenic strains in two forms (one the haptenic, free ECA; the other one, the LPS-linked form, see Sect. VII. B), and such strains were repeatedly selected for preparative ECA extraction (*Kunin*, 1963; *Hammarström* et al., 1971).

Even in ECA-nonimmunogenic strains one usually finds ECA in different aggregation forms with differing immunologic properties (*Whang* et al., 1971 a), see Sect. VII. A. In addition, the important question whether there exists only a single common antigen, which can be demonstrated with *E. coli* 014 antisera using the passive hemagglutination test, is not unequivocally answered (*Conley* et al., 1976). Furthermore, it was repeatedly observed that ECA can loose some of its characteristic properties during its isolation, e.g., the erythrocyte-sensitizing capacity. For instance, this property is lost when ECA is subjected to mild alkali treatment (0.25 *N* NaOH, 37° C, 1 h) (*Whang* et al., 1971 a) or by incubation with phospholipase A (*Marx* and *Mayer,* 1974). The latter enzyme has also been reported to occur in ECA-containing strains (*Hayaishi* and *Kornberg,* 1954). ECA modified by either alkali or phospholipase A treatment is still active in hemagglutination inhibition and agar gel precipitation tests (see Fig. 1 a).

In addition, the erythrocyte-modifying capacity of ECA (as well as of other bacterial antigens) is inhibited by various substances, including protamine, histone, polymyxin B, and neomycin (*Gorzynski* and *Neter,* 1966), and also by normal rabbit antiserum. Also, sera from other species of higher animals are active as inhibitors in contrast to sera from lower animals, which show much less or no inhibitory activity (*Praino* and *Neter,* 1977). *Springer* et al. (1970) showed that an isolated membrane component, characterized as a negatively charged lipoglycoprotein, acts as an inhibitor to the attachment of endotoxin and ECA to erythrocytes. A certain degree of specificity is evident from the fact that this erythrocyte component reacts far more effectively with LPS and ECA than with Vi antigen and other antigens from gram-positive bacteria in preventing attachment to red blood cells. Because of this specificity for LPS, it was referred to as LPS receptor. The high affinity of the LPS receptor for endotoxin and ECA is astonishing because both receptor and bacterial antigens are strongly negatively charged, the former on account of its content in *N*-acetylneuraminic acid. In this respect it can be assumed that the (basic) inhibitors complex with endotoxin and ECA and thus affect the affinity to erythrocytes.

Polymyxin B, however, does not alter the receptor in the erythrocyte membrane since a pretreatment of erythrocytes with the antibiotics does not interfere with a subsequent reaction (*Gorzynski* and *Neter,* 1966).

Regarding ECA isolation, it was a major breakthrough when *Suzuki* et al. (1964a) discovered that ECA and LPS from nonimmunogenic strains can be separated by a rather simple fractionation with 85% aqueous ethanol: ECA is soluble in ethanol, LPS remains in the insoluble fraction. Most of the later-developed extraction procedures make use of the observed ethanol solubility of ECA (*Marx* and *Petcovici*, 1975; *McLaughlin* and *Domingue*, 1974).

Another most valuable observation was the precipitating capacity of some, but certainly not all, ECA antisera with high hemagglutination titers (*Mayer* and *Schmidt,* 1971; *Johns* et al., 1973; *Whang* et al., 1973). Precipitation of ECA is independent of its erythrocyte-modifying capacity and is also observed with alkali-treated and phospholipase-treated preparations (*Kuhn,* unpublished). Gel precipitation is now of considerable value in following ECA during the extraction process and for testing the uniformity of isolated material.

ECA-negative mutants are now available from *Salmonella* and *E. coli* (*Mäkelä* et al., 1974; *Schmidt* et al., 1976b), however, of particular value are ECA-negative mutants of otherwise immunogenic strains: *E. coli* 014:K7 and *E. coli* 08^-:K27^- (*Kiss* et al., 1978). These can be used for serum absorptions, and thus allow the preparation of monospecific ECA antisera, which can be used for semiquantitative tests (radial immunodiffusion, rocket immunoelectrophoresis etc.). ECA-negative strains are also important as negative controls.

B. Earlier Extraction Procedures and Chemical Studies

The earlier work on ECA extraction and chemical studies on ECA-containing fractions has been reviewed in detail (*Mäkelä* and *Mayer,* 1976). The conclusions reached by these studies varied widely and were derived from different approaches to the problem. Nevertheless, these studies provided some information which was very useful for subsequent work and are discussed here in relation to more recent developments.

Kunin (1963) applied the hot phenol–water method for extraction of ECA from *E. coli* 014 and some other enterobacterial wild-type strains and R mutants. The resulting aqueous phase was precipitated with ethanol (ten volumes) and the precipitate was subjected to DEAE-cellulose chromatography. ECA and O (R) antigens were screened by hemagglutination inhibition and were found to be partly separable from each other. It was observed that ECA activity is not destroyed by either periodate oxidation or by trypsin digestion. The isolated ECA had lost the capacity to modify erythrocytes and to elicit an ECA immune response. Chemical analysis of three ECA fractions from *E. coli* 014 were reported and showed low and varying contents of protein, 12%–20% reducing sugar, mostly hexosamin (8%–17%). Lipid was not demonstrable and phosphorus was low (0.5%). It was noticed that ECA is more resistent to acid hydrolysis (0.1 *N* HCl, 60° C) than O antigen of *Salmonella typhosa* (*Kunin,* 1963).

Hammarström et al. (1971) also used *E. coli* 014 for ECA isolation and phenol/water for the extraction of bacteria. The aqueous phase material containing O (R4, see Sect. V. B) and ECA specificity was first passed over a Sepharose 4B column and showed the presence of appreciable amounts of glucose, galactose, heptose, and KDO (2-keto-3-deoxy-octonate) in addition to some glucosamine and O acetyl. Treatment of the material with alkali removed O acetyl, but did not affect the serological ECA specificity. The alkali-treated material was hydrolyzed with 1% acetic acid (1.5 h, 100° C), a method used to split the ketosidic linkage between lipid A and sugar chains in LPS (*Lüderitz* et al., 1971). The acid-soluble fraction was passed over a Sephadex G-50 column using a pyridine–acetic acid buffer as solvent. A major peak appeared in the molecular weight range of $2–3 \times 10^3$. This peak inhibited precipitation of alkali-treated 014 LPS by antibodies to ECA from a *E. coli* 014 antiserum, but it also inhibited hemagglutination between ECA antibodies and erythrocytes coated with *E. coli* 08 LPS. The chemical analysis corresponded in composition to the core region of *E. coli* 014 LPS in detecting heptose, KDO, glucose, and galactose, although glucosamine was not present in similar quantities.

This result is consistent with the finding of *Kiss* et al. (1978) with an ECA-immunogenic R1 strain. However, it was not recognized that 014 (R4) specificity and ECA specificity are different antigenic entities; a result only obtainable by studies with ECA-negative mutants of ECA-immunogenic strains (*rfa*L or *rfe* mutants, see Sect. V. B). Of special interest is the finding that ECA and 014 antigenic specificities are not separable by hydrolysis with 1% acetic acid at 100° C.

The discrepancies between the findings of *Kunin* and *Hammarström* when both using *E. coli* 014 were due to the fact that the latter examined the LPS-linked ECA, whereas *Kunin* dealt with a mixture of free and LPS-linked ECA.

Johns et al. (1973) used *Salmonella typhosa* 0:901 for extraction, chromatography on Sephadex G-200, and preparative gel electrophoresis for purification. Purified ECA was active in inhibiting passive hemagglutination of erythrocytes coated with crude ECA preparations and could be demonstrated in agar gel precipitation, where a single precipitation band with a strongly anodically moving antigen (pH 8.6) was observed. The purified material, however, failed to coat erythrocytes for hemagglutination and immunogenicity was not observed in rabbits. Chemical analysis indicated carbohydrate to be the major constituent of ECA (mostly hexoses, some hexosamine).

It is not certain whether the antigen isolated by Johns is identical with the ManNAcUA/GlcN polymer of *Männel* and *Mayer* (1978a) and *Lugowski* and *Romanowska* (1978), although the lack of the precipitation and hemagglutination in *rfe* mutants of *Salmonella minnesota* (chemotypes Rc–Re) agrees with the studies by *Mäkelä* et al. (1974) on the distribution of ECA.

Other studies (*McLaughlin* and *Domingue,* 1974) made use of the ethanol solubility of ECA for extraction. Chemical analysis shows, however, that the material obtained is heavily contaminated with protein and RNA.

The studies, while not revealing the chemical nature of ECA, do reveal some of its characteristics: It is heat-stable, its antigenicity withstands heating to 120° C (*Whang* and *Neter,* 1962), and it is not destroyed by trypsin or pronase

digestion (*Kunin,* 1963). ECA was found to be highly negatively charged and more stable towards acid hydrolysis than LPS (*Kunin,* 1963).

ECA is formed – although to a lesser extent – by Enterobacteriaceae in a completely synthetic medium (*Gorzynski* and *Neter,* 1970), showing that ECA biosynthesis is independent from special factors of the culture medium.

An interesting observation was made by *Whang* and *Neter* (1964) by chance: they found that *Pseudomonas aeruginosa* produces a factor, presumably an enzyme, which destroys selectively ECA from various strains. It also destroys ethanol-soluble ECA after separation from the ethanol-insoluble LPS. The ethanol-insoluble ECA (LPS-linked ECA of *E. coli* 014) is, however, not affected by the *Pseudomonas* factor. The factor was also detected in a psychrophilic *Pseudomonas* strain (*Whang* and *Neter,* 1965a). Heating for 30 min at 56° C does not destroy its activity, but heating for 10 min at 100° C does. The inactivation of ECA is more rapid at 56° C than either at 37° C or 4° C. The *Pseudomonas* factor will probably be of importance for analytic studies with isolated ECA. Studies in this direction are presently being carried out in our group.

C. Recent Isolation Procedures: Chemical Identity

The hitherto reviewed methods of ECA extraction yielded only semipurified preparations, which were active in inhibition of ECA hemagglutination systems, but had nevertheless lost some of the characteristic properties of ECA, namely the erythrocyte-sensitizing capacity and/or the immunogenicity in experimental animals.

The first report on ECA preparations highly enriched for ECA which had retained all ECA characteristics was published in 1975 (*Marx* and *Petcovici,* 1975). They used the Ra type mutant TV 149 of *S. typhimurium* as source of ECA. Extraction of bacterial wet mass was carried out under constant agitation with 96% ethanol at 60° C for 20 min. The resulting solution was cooled to 20° C and centrifuged. The supernatant was concentrated to a small volume, and the viscous fluid redissolved with 85% aqueous ethanol. The ethanol-soluble material was mixed with three volumes of acetone; the resulting sediment was dissolved in water and passed over a Sephadex G-75 column. ECA activity appeared in the void volume (V_0 fraction).

The yield of ECA was small (0.0025% of bacterial wet weight). Chemical investigations revealed the presence of sugars (3.4% glucose, 4.1% glucosamine), protein (15%), fatty acids (0.6% myristic, 14.8% palmitic, and 4.9% stearic acid), and in addition glycerol (1.3%), phosphorus (1.9%), and ethanolamine (not quantitated). The absence of β-hydroxymyristic acid indicates the absence of LPS; other criteria of purity were not given. The material was immunogenic in rabbits and retained its erythrocyte-coating capacity. The presence of constituents characterizing a cephalinlike phosphoglyceride, in addition to the observed action of phospholipase A, was taken as proof that ECA has basically the structure of a L-phosphoglyceride (*Marx* and *Petcovici,* 1975). On incubation with phospholipase A, ECA did not only loose its erythrocyte-coating ability, as described earlier (*Marx* and *Mayer,* 1974), but also its hemagglutination-

inhibiting capacity, indicating that the serological determinants were also affected by phospholipase A. The action of the enzyme was accompanied by release of about 50% of the fatty acids, a value in agreement with a phosphoglyceride structure.

Another extraction and purification method was developed by *Männel* and *Mayer* (1978a; *Männel,* 1976) on the basis of an earlier observation that LPS extracted by the hot phenol–water method of *Westphal* et al. (1952) is mostly contaminated with ECA (see footnote page 102) whereas LPS extracted by PCP is not (*Mayer* and *Schmidt,* 1971).

Phenol-killed bacteria of *Salmonella montevideo* SH94 were first extracted with 45% aqueous phenol at 68° C; the resulting aqueous phase, after dialysis and lyophilization, was then treated with the PCP mixture (2/5/8, v/v) according to *Galanos* et al. (1969). After removal of the volatile chloroform and petroleum ether under reduced pressure, LPS was precipitated by adding several drops of water to the resulting phenol phase. While LPS precipitates, ECA remains in solution and can be recovered from the phenol phase by extensive dialysis and lyophilization. The resulting material was resuspended in water and centrifuged at 105,000 × g for 4 h. Although ECA activity was present in both fractions, pellet and supernatant, only the latter was taken for further purification of ECA. The lyophilized material obtained from the supernate fraction was applied to a DEAE column, previously equilibrated with 0.5 *M* ammonium acetate, and was eluted stepwise with a buffer of increasing concentration of ammonium acetate (0.5, 1.0, and 1.5 *M*) in methanol. ECA was recovered from the middle fraction and rechromatographed in the same column with a buffer of 0.9 *M* ammonium acetate/methanol. In addition, fractionation of the material with 85% aqueous ethanol or electrodialysis of the final product (*Galanos* and *Lüderitz,* 1975) were sometimes carried out, but no substantial further enrichment of ECA was noticed.

Chemical analysis of the isolated product revealed that ECA, in contrast to all previous reports, is an amino sugar heteropolymer mainly composed of *N*-acetyl-D-glucosamine and *N*-acetyl-D-mannosaminuronic acid, being partly esterified by small amounts of palmitic and acetic acid residues and accounting for about 60%–63% of the lyophilized material (see Table 2 for the overall analysis). Methylation and analytic examination of the partly methylated hydrolysis products by gas-liquid-chromatography/mass spectrometry in combination with oligosaccharide analysis showed that ECA is composed of a linear chain of alternating units of 1.4-linked *N*-acetyl-D-glucosamine and *N*-acetyl-D-mannosaminuronic acid (as depicted in Fig. 2). Preliminary data in nuclear-magnetic-resonance spectroscopy point to the presence of one α- and one β-glycosidic linkage (*Männel* and *Mayer,* 1978a). The presence of an additional, still unknown lipid constituent that may account for the missing material (30% on a weight basis) and the low solubility of the isolated antigen in water has been suggested. The molecular weight of the portion of ECA readily soluble in methanol was determined by the method of Yphantis to be 2700. This low molecular weight of nonaggregated ECA is consistent with a structure having eight to ten amino sugars in a linear chain. In aqueous solution, ECA certainly forms micelles of much higher molecular weight and this is probably the reason for the observa-

Table 2. Chemical analyses of ECA from *Salmonella montevideo* and *Shigella sonnei*

	S. montevideo SH94[a]	Sh. sonnei 9773 (phase I)[b]
Amino sugars total	45–50	62.9
(D-glucosamine)	(23–25)	(19.5)
(D-mannosaminuronic acid)	(23–25)	(43.4)
N-Acetyl	11.7	15.7
O-Acetyl	1.3	3.6
Fatty acids total	2.5	3.2
(Palmitic acid)	(2.0)	(+)
($C_{18:1}$)	(±)	(+)
Phosphate	0.47	P 0.5
Neutral sugars	absent	1.6
Amino acids	absent	4.0
Total	70%	90%
Solubility in water (25° C)	low (0.3%)	good (10%)
Yield[c]	0.3%	0.2–0.25%

[a] Data from *Männel* and *Mayer*, 1978a.
[b] Data from *Lugowski* and *Romanowska*, 1978.
[c] % of bacterial dry weight.

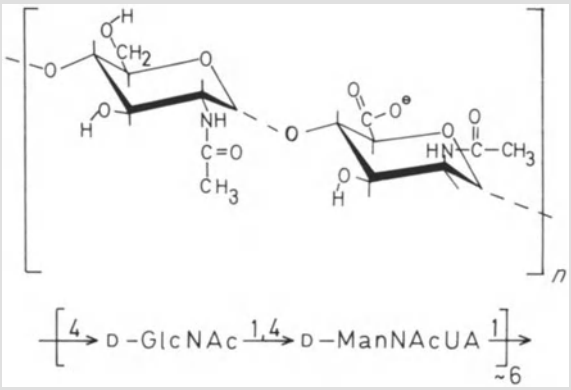

Fig. 2. Proposed structure for the sugar moiety of enterobacterial common antigen obtained from *Salmonella montevideo* SH94. *Männel* and *Mayer*, Eur. J. Biochem. *86*, 368, 1978a

tion that aqueous ECA solutions can be dialyzed without significant loss of material.

Isolated ECA is nonhomogeneous judging from the precipitation pattern observed in immunoelectrophoresis (see Fig. 1), but after alkali treatment a more uniform and distinct line is observed (*Männel* and *Mayer,* 1978b). This effect is most probably due to the splitting of ester-linked fatty acids (palmitic and acetic acids), indicating that the inhomogeneous precipitation is probably caused by the micellar form of ECA. A similar change in precipitation is also observed after treatment with phospholipase A (*Kuhn,* unpublished).

A similar situation has been described for lipoteichoic acids, a group of amphiphilic antigens of gram-positive bacteria, in which fully acylated micellar

forms as well as deacylated monomer forms coexist in the gram-positive cell wall. In addition, the lipid part is easily split off during extraction with trichloroacetic acid, and the resulting teichoic acid loses essential biologic properties (*Wicken* and *Knox,* 1975). A glycerol lipoteichoic acid from *Streptococcus pyogenes* contains a small amount of fatty acid (1.5%). With this antigenic material too the fatty acid moiety proved to be essential for erythrocyte sensitization and for micelle formation (*Rudczynski* and *Jackson,* 1978).

Since these results concerning the chemical nature of ECA were different from results of all previous studies, a careful characterization of the material has been made. First, it was proven that a similar GlcN/ManNUA polymer could be extracted from other enterobacterial wild-types and mutants. It has now been isolated from *Citrobacter* (1658), *Salmonella arizonae* (F 628), *S. minnesota* R 1, *E. coli* (F870), *Proteus mirabilis* (1959), *P. rettgeri* and from mutants with defects in glucose, galactose, and mannose biosynthesis (UDP-phosphorylase-less mutant Gal 23 K^-, UDPGal-4-epimerase-less mutant PL-2 of *E. coli* K-12, and phosphomannose-isomerase-less mutant of *S. typhimurium*), to name but a few (*Mäkelä* and *Mayer,* 1976; *Männel* and *Mayer,* 1978a). Furthermore, not a single strain identified by serological analysis as being ECA-positive was found to lack this aminosugar polymer completely, although its concentration may vary.

Final proof of the identity of the isolated material with the *Kunin* antigen came from a study of various related strains of *S. montevideo* and *S. typhimurium* with defined genetic defects in *rfe/rff* genes and their recombinants (see Sect. V). A good correlation was found between serologically determined presence of ECA and the chemically determined presence of the rare sugar constituent D-mannosaminuronic acid (*Männel* et al., 1978). Strains classified by serological techniques as ECA-positive, ECA-negative or ECA trace were found to possess the expected amounts of mannosaminuronic acid in the ECA-enriched phenol-phase material after combined phenol/water–PCP extraction.

The isolated ECA of *S. montevideo* was highly active in coating erythrocytes for agglutination with antisera specific for ECA and in inhibition of the corresponding hemagglutination systems. Attempts to immunize rabbits with the isolated soluble ECA were unsuccessful. A transient antibody response was observed in mice when small amounts of the isolated ECA (1–10 μg) were administered i.v. The titers fell off rapidly and the NMRI mice became unresponsive to a booster reaction with the same antigen (*Männel* and *Mayer,* 1978b). However, high ECA specific titers were elicited in rabbits and NMRI mice when immunization was carried out with the antigen attached to acetic acid-hydrolyzed *S. minnesota* R 595 bacteria. This method, first introduced by *Galanos* et al. (1971) for engendering lipid A antibodies, makes use of the fact that the antigen is presented in an accessible form and together with lipid A and lipoprotein, both of which are potent mitogens (*Melchers* et al., 1975; *Andersson* et al., 1973). *S. minnesota* R 595 is an ECA-negative mutant due to an *rfe* defect (*Mäkelä* et al., 1974), and consequently no antibodies to ECA were found in control animals immunized with uncoated acetic acid-treated bacteria.

It was further observed with the isolated ECA that esterification of the carboxylic group of D-mannosaminuronic acid leads to a reversible loss of

its precipitating capacity, proving that this aminuronic acid is an essential part of the serological determinant of ECA. Thus, considering the rare occurrence of D-mannosaminuronic acid in gram-negative bacteria, where it has only been found as a constituent of the K 7 and K 56 capsular antigen of *E. coli* (*Mayer,* 1969; *Flemming,* 1972), its easy detection by paper electrophoretic methods can serve as a good indicator of ECA presence. It is necessary, however, to confirm these observations by serological techniques.

Examination of the ECA material isolated by *Marx* and *Petcovici* (1975) indeed showed the presence of significant amounts of mannosaminuronic acid (see, Discussion in *Marx* and *Petcovici,* 1975), showing that our material was at least present to some extent in the isolate of these authors.

Immunoelectrophoretic examinations showed an additional faint precipitation line (Fig. 1 b) with isolated and carefully purified ECA in some but not all precipitating antisera (*Männel* and *Mayer,* 1978 b). This poses some questions with regard to the existence of serologically distinct but nevertheless similar antigens. This was recognized earlier by *McCabe* and *Johns* (personal communication, discussed in *Mäkelä* and *Mayer,* 1976) and *Conley* et al. (1976). It should be noted, however, that absorption of these precipitating antisera with ECA-negative mutants (genetically *rfe*$^-$ as well as *rff*$^-$) produced no change in the precipitation pattern, indicating that all precipitating material is absent in ECA-negative mutants of both types. Regarding the function of the *rff*-genes, which determine enzymes of the ManNAcUA biosynthesis (*Lew* et al. 1978), it seems that this additional line might be caused by a structurally related antigen (see following paragraph), e.g., a partly O-acetylated ECA, as discussed by *Lugowski* and *Romanowska,* 1978).

The results on the chemical nature of ECA were recently confirmed, at least basically, by studies of *Lugowski* and *Romanowska* (1978) using *Shigella sonnei* phases I and II as sources of ECA. They developed a new extraction method starting from a sonicate of bacteria dissolved in phosphate buffer of pH 7.

The ultrasonic disintegration was carried out in the presence of EDTA and sodium azide (12 min), then lysozyme was added and the sonication repeated for 6 min. After overnight incubation under constant stirring, the solution was centrifuged at 40,000 × g. The supernate was mixed with ethanol to a final concentration of 85%, and ECA was then precipitated by acetone at 20° C. The pellet obtained after the initial centrifugation at 40,000 × g was extracted with water and the supernatant of a 5000 × g centrifugation-run was also subjected to the ethanol fractionation outlined above. The combined extracts were further purified by silica gel chromatography. A first elution with butanol/acetic acid/water (6/2/2, v/v/v) removed material without ECA activity; a second elution with ethanol/water (7/3, v/v), however, yielded ECA material which was applied to a Sephadex LH-20 column for a final purification. Methanol was used for elution and fused rocket electrophoresis (with 014 antiserum) for ECA detection. The elution profile revealed that ECA was recovered in tubes 23–28, i.e., in a relatively sharp zone.

Purified ECA was recovered in 0.2%–0.25% yield of bacterial dry mass, a value quite similar to that obtained by *Männel* and *Mayer* (1978 a) with

Salmonella montevideo (0.3%), but 100 times higher than that reported by *Marx* and *Petcovici* (1975).

The material obtained by *Lugowski* and *Romanowska* showed two precipitin lines in immunodiffusion using anti-*E. coli* 014 serum and, as expected, no reaction with *Shigella sonnei* phase I antiserum, although the material was derived from this strain. In immunoelectrophoresis a birdwing precipitation pattern was observed reminiscent of the finding of *Mayer* and *Schmidt* (1971) and *Männel* and *Mayer* (1978b) using isolated ECA (see Fig. 1). In addition the material was active in coating horse erythrocytes and in inhibition of an ECA hemagglutinating system. Immunization of rabbits (five injections) with increasing doses of the antigen (0.2–3 mg) gave moderate hemagglutination titers of 320–1280. Considering the high amount of antigen applied, the immunogenicity of the isolated material was poor.

The results of the chemical investigation are compiled in Table 2 together with the previously published data of *Männel* and *Mayer* (1978a). Again, ECA was recognized as an amino sugar polymer built of *N*-acetyl-D-glucosamine and *N*-acetyl-D-mannosaminuronic acid, partly esterified by acetic and palmitic acids and in addition C18:1 (not oleic acid). Comparison of the chemical composition described for the two preparations shows great similarities, but also obvious differences. Firstly, the total recovery of constituents is higher with the *Shigella sonnei* material (about 90%) than with *Salmonella* (70%). ECA of *Shigella* is of fairly good water solubility (about 10% vs 0.3% reported for the solubility of ECA from *Salmonella,* see Table 2). In addition, a ratio of 2:1 was determined (by extrapolation) for ManNAcUA/GlcNAc, whereas a 1:1 ratio was reported for *Salmonella* SH94. In both cases, however, the quantitation of the aminuronic acid had to be made indirectly because of the acid lability of ManNUA (*Perkins,* 1963). Aside from these differences, the two preparations contain the same constituents, and the serological behavior in immunoelectrophoresis before and after alkali treatment is the same.

Whether some of the differences, such as in water solubility, are matters of different aggregation or cross-linking alone or caused by a different make up of the sugar chain (disaccharide repeating unit vs trisaccharide repeating unit) has to be explored. It has to be taken into consideration, however, that ECA of *Salmonella* could be linked to a non-LPS carrier, which could alter the solubility of the antigen and could also account for the missing 30% of material (*Männel* and *Mayer,* 1978a). In ECA-immunogenic strains the ethanol-soluble ECA moiety is known to differ in immunogenicity from the ethanol-soluble ECA of non-immunogenic strains (*Whang* et al., 1972b; *Suzuki* et al., 1966) (see Sect. VII. A), and *Shigella sonnei* phase II, which is formed readily from phase I, is an ECA-immunogenic strain. It will be discussed later (Sect. VII. B) that LPS of ECA-immunogenic strains can also serve as a high molecular carrier of ECA.

The confirmation of the main data of *Männel* and *Mayer* (1978a, b) regarding the chemical identity of ECA by the studies of *Lugowski* and *Romanowska* (1978) is of special interest since ECA was isolated from a strain of another genus and by an independent isolation route.

A very recent paper by *Measel* (1978) describes isolation of ECA from

ethanol-soluble extracts of heated strains by affinity chromatography using covalently linked ECA antibodies on inert beads. ECA was released from the column after thorough washing with PBS by salt treatment. The material was serologically active, but did not induce antibody formation. Isoelectric focusing showed its i.p. to be approximately 5.0. Preliminary analyses showed only a small content of amino acids in the isolated material. It would be of interest to compare the isolate regarding its content of amino sugars, but these analyses are not yet available.

V. Genetic Determination

A. Genes Involved in ECA Biosynthesis

It has recently been shown that two gene clusters, *rfe* and *rff*, are involved in ECA biosynthesis (*Mäkelä* et al., 1970; *Mäkelä* and *Mayer*, 1974; *Mäkelä* et al., 1974; *Schmidt* et al., 1976b). Both the *rfe* and *rff* genes are located near to the isoleucine/valine gene *ilv* at position 84 of the *Salmonella* chromosomal map (*Sanderson* and *Hartman,* 1978). The *rfe* region is also required for the synthesis of O-specific polysaccharide chains of LPSs in certain *Salmonella* species, but not in others (*Mäkelä* and *Mayer*, 1974; *Mäkelä* et al., 1976).

In addition, in *Salmonella* bacteria of serogroup B some undefined parts of the *rfb* gene cluster, which is determining the O-specific polysaccharide structure, are required for ECA biosynthesis (*Mäkelä* et al., 1976). Moreover, other LPS genes are involved in the linkage of ECA to LPS in certain ECA-immunogenic strains (*Schmidt* et al., 1976a). Thus the involvement of LPS genes in the production and immunogenicity of ECA makes it necessary to review briefly some genetic aspects of the biosynthesis of LPS (Fig. 3).

The O-antigenic LPS consists of an O-specific polysaccharide linked to a core oligosaccharide, which in turn is bound to lipid (*Lüderitz* et al., 1971; *Jann* and *Westphal*, 1975; *Lüderitz* et al., 1978). The biosynthesis of the core and the O-specific chains is under control of *rfa* and *rfb* genes, respectively (*Stocker* and *Mäkelä*, 1971). Most of the *rfa* genes are clustered in the *cys*E-*pyr*E region (Fig. 3), whereas the *rfb* genes form a cluster closely linked to the *his* operon. In a still unknown way the *rfe* genes are also involved in the synthesis of O-specific chains in *S. minnesota* (021, serogroup L), in *S. montevideo* (06,7; serogroup C_1), and in several *E. coli* serotypes (*Mäkelä* and *Mayer,* 1974; *Schmidt* et al., 1976b). A mutation in this region results in rough (R) mutants which phenotypically resemble *rfb*-defective mutants. Moreover, it could be shown that this mutation is also responsible for the lack of ECA in these strains. It was concluded that *rfe* forms a cluster of genes involved in O-chain synthesis of certain serotypes as well as in ECA production. On the other hand, *S. typhimurium* (serogroup B) also has *rfe* genes required for the synthesis of ECA, but not for the synthesis of the 04,12-type B polysaccharide (*Mäkelä* and *Mayer*, 1974).

The *rfe* gene product has not yet been identified. One can assume, however, that the *rfe* genes are somehow involved in a step common to the synthesis

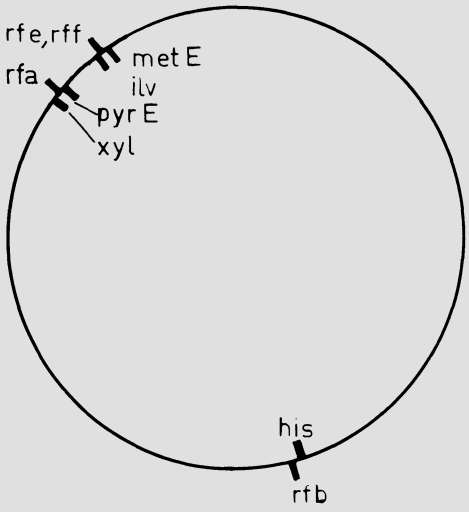

Fig. 3. Simplified chromosome map of Salmonella showing the positions of rf genes. (*Mäkelä* and *Mayer*, Bacteriol. Rev., 1976)

of ECA, several O-specific chains, and the T1 polysaccharide (*Stocker* and *Mäkelä*, 1971). This may concern a carrier molecule needed for the assembly of these polymers (*Mäkelä* et al., 1970). The carrier molecule produced by these strains should be different from the well-known antigen carrier lipid of *Salmonella* of group B (*Robbins* and *Wright*, 1971), since these bacteria do not require the presence of *rfe* genes for the synthesis of their O-specific polysaccharides. In fact, recent observations indicated that probably a different mode of synthesis of O chains exists in *S. montevideo* of serogroup C_1 (*Gmeiner*, 1975) and *E. coli* 09 (*Kanegasaki* and *Jann*, 1978).

Another type of mutation, which blocks the synthesis of ECA but does not affect the O-specific polysaccharides, was found accidentally in *S. minnesota* (*Mäkelä* et al., 1974). The site of this mutation was closely linked to the *ilv–rfe* region, and the genes concerned were called *rff* (see Fig. 3). Very recently it was shown that *rff* genes are involved in the synthesis of UDPManNAcUA, the activated form of D-mannosaminuronic acid, a main constituent of ECA (*Lew* et al., 1978). The biosynthetic pathway leading to ManNAcUA (Fig. 4) was revealed in *E. coli* by *Ichihara* et al. (1974) and *Kawamura* et al. (1975) and was also recently demonstrated in *S. typhimurium* (*Lew* et al., 1978). Moreover, it was established that genes of the *rff* cluster of *S. typhimurium* control the function of an epimerase, *rff*E, as well as that of a dehydrogenase, *rff*D, which are involved in the two reaction steps leading to UDPManNAcUA, as schematically presented in Fig. 4. It was shown by measuring the enzyme activities that most of the *rff* mutants, serologically characterized as ECA-negative (*Mäkelä* et al., 1976), had defects in the NAD^+-dependant dehydrogenase, whereas additional defects in the epimerase were seen only in a single strain (*Lew* et al., 1978).

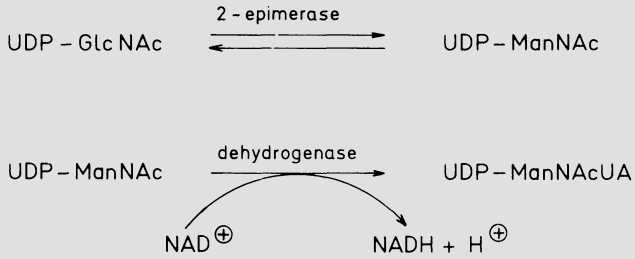

Fig. 4. Biosynthesis of *N*-acetylmannosaminuronic acid (according to *Kawamura* et al., 1975)

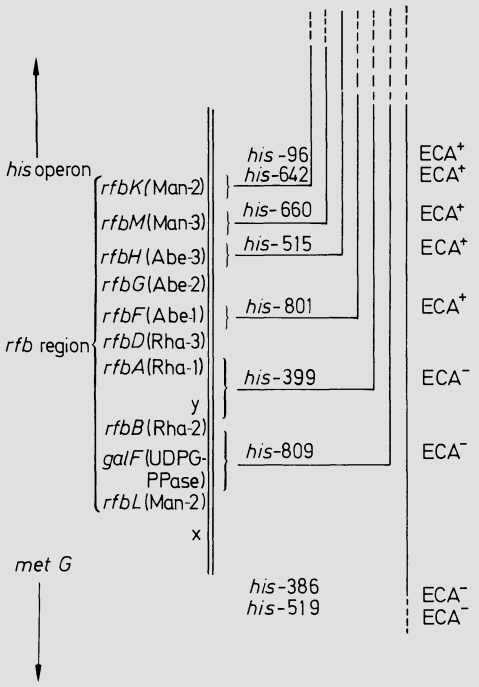

Fig. 5. Schematic map of the *rfb* region of *Salmonella typhimurium* and the extent of *his* deletions (*Nikaido* et al., 1967). The presence or absence of ECA is also indicated (*Mäkelä* et al., 1976). The enzymes determined by the *rfb* genes are shown in parentheses (*Man-2*, phosphomannomutase; *Man-3*, GDP-mannose pyrophosphorylase; *Abe-3*, "CDP-abequose synthetase"; *Abe-2*, GDP-glucose oxidoreductase; *Abe-1*, GDP-glucose pyrophosphorylase; *Rha-3*, "TDP-rhamnose synthetase"; *Rha-1*, TDP-glucose pyrophosphorylase; *Rha-2*, TDP-glucose oxidoreductase; *x*, *y*, unidentified functions). (*Lew* et al., 1978)

In *S. typhimurium* ECA synthesis is additionally determined by parts of the *his*-linked *rfb* gene cluster (*Mäkelä* et al., 1976). This became evident when a series of *S. typhimurium* R mutants with various lengths of deletions in their *his–rfb* regions (*Nikaido* et al., 1967) were tested for presence of ECA. Strains with long deletions were ECA-negative and those with shorter deletions were ECA-positive (Fig. 5). However, when long *his–rfb* deletions were cured by

introduction of the wild-type *his–rfb* region the resulting hybrids produced smooth (S) type LPS, but were still ECA-negative. It soon became evident that these long deletion mutants had undergone a second mutation which was the *rff* type mentioned above. When in ECA-negative deletion mutants the rff^- was replaced by rff^+, the resulting hybrids produced traces of ECA only. Moreover, they showed a reduced viability and an increased sensitivity to sodium dodecyl sulfate. Upon storage these hybrids soon accumulated again mutations in the *rff* genes leading to the ECA-negative phenotype, and again displayed normal levels of detergent sensitivity (*Mäkelä* et al., 1976). Similarly, the introduction of the group C *rfb* from *S. montevideo* to wild-type *S. typhimurium* resulted in smooth 06,7 hybrids that showed a dramatically reduced synthesis of ECA. This clearly demonstrates that in Salmonella of group C_1, in contrast to group B Salmonella, all genetic determinants of ECA production so far recognized are assembled in the *ilv*-linked *rfe–rff* region and none are linked to *rfb* (*Mäkelä* and *Mayer*, 1974).

B. Genetic Basis for ECA Immunogenicity of Strains

For immunogenicity in the bacterial cell the ECA immunodeterminant must be linked to the LPS R core (*Mayer* et al., 1972; Fig. 6). As described in Sect. VII.B, the presence of distinct LPS core types such as R1 or R4 is essential for ECA to be immunogenic (*Mayer* and *Schmidt*, 1971; *Schmidt* et al., 1974).

Mutation in	Mutation blocking	LPS - structure	ECA presence in bacteria	ECA immunogenicity
—	—	O-chains Core Lipid A	+	—
rff	ECA synthesis		—	—
rfb	O-chain synthesis	ECA	+	+
rfe	?????		—	—
rfaL	ECA and O-chains translocation		+	—
rfa	Core synthesis		+	—

Fig. 6. ECA and LPS characteristics after mutation of different rf genes. Studied in *Escherichia coli* strains with the LPS core type R_1 or R_4

Thus a *rfa* mutant of *E. coli* 014 (R4) having an incomplete core was found to be nonimmunogenic (*Marx* and *Petcovici*, 1976).

R1 and R4 mutants with a defective *rfa*L gene, which is part of the *rfa* gene cluster and which is involved in the translocation of O chains to the core, were not immunogenic although they produced ECA and had a complete core (*Schmidt* et al., 1976a). Therefore it is suggested that the function of the *rfa*L gene is required for translocation of ECA onto the LPS core (see Fig. 5) and hence is essential for ECA to be immunogenic. Thus ECA of immunogenic strains has in common with other polymers, such as O and T1 polysaccharides, linkage to the complete LPS core, and this translocation process needs the function of the *rfa*L gene.

VI. Localization of ECA in the Bacterial Cell

Judging from the facts that upon immunization with whole bacterial cells ECA antibodies are produced only with a few enterobacterial strains, e.g., *E. coli* 014:K7, and that ECA-containing cells are not agglutinated by ECA antibodies, it might have been assumed that ECA is not sufficiently exposed on the surface of Enterobacteriaceae or that it is not even a constituent of the outer membrane. On the other hand, phagocytosis and bactericidal activity require an antibody–surface interaction and both activities have been demonstrated with ECA-containing strains in the presence of antibodies (*Domingue* and *Neter*, 1966b), although in the case of bactericidal activity this was only so with ECA-immunogenic *E. coli* 014. Also, the ease with which ECA is liberated into the medium (*Kunin* et al., 1962) and the ease with which ECA antibodies can be absorbed by bacteria suggests localization on the cell surface. That ECA is indeed localized in the outer cell membrane was shown by immunofluorescent techniques (*Aoki* et al., 1966). Again, it was the immunogenic *E. coli* 014 which showed the most intense fluorescence, with the stain homogeneously distributed over the surface. Other strains stained less intensively and showed more irregular spotty distribution of fluorescence (*Aoki* et al., 1966). The same results—intense and evenly distributed fluorescence with ECA-immunogenic strains, weaker and granular distribution in nonimmunogenic strains—were obtained when monospecific ECA antisera were used in a sandwich technique with fluorescent goat–antirabbit IgG (*Rinno*, 1978).

It has recently been reported (*Marx* et al., 1977) that bacterial agglutination by ECA antibodies can be observed with enterobacterial R mutants. This agglutination, also observable with monospecific ECA antisera, is not solely dependent on the presence of immunogenic ECA; nevertheless the highest titers were obtained with ECA-immunogenic strains. This suggests the importance of the steric exposure of ECA for agglutinability and might be the reason for the lack of agglutination observed with all enterobacterial S forms, which is in accordance with earlier reports (*Kunin*, 1963; *Neter* and *Whang*, 1972). It also suggests that O chains partly cover the free (unbound) ECA, thereby making it less accessible for ECA agglutinins (*Marx* et al., 1977).

With monospecific ECA antisera a direct demonstration of ECA in the outer membrane was recently made possible by using ferritin-conjugated ECA antibodies or antirabbit IgG antibodies. Ferritin-antibody conjugates were incubated with three closely related mutants of *E. coli* 08:K27$^-$, possessing either the immunogenic form of ECA, the haptenic form, or neither. Freeze-etch studies showed a fairly dense labeling of the first two mutants in contrast to a very weak labeling in the case of the ECA-negative strain, the latter probably being caused by unspecific absorption (*Rinno* et al., 1978; *Kiss* et al., 1977; *Golecki*, unpublished).

The most direct attempt to localize ECA in the bacterial cell was made by *Domingue* and *Johnson* (1974) and *Johnson* et al. (1976). They prepared different fractions, including whole outer membrane and cytoplasmic membrane, by cell rupture in a French pressure cell and subsequent ultracentrifugation and fractionation by electrophoresis. ECA was detected in almost all fractions of three different enterobacterial strains, but to various extents. The authors came to the conclusion that ECA is located in the cell envelope, but not necessarily exclusively since it was also demonstrated in vesicular membranous components and in the soluble fraction. In *E. coli* K-12, ECA seemed to be equally located in the outer and inner membrane (*Johnson* et al., 1976). The possibility that ECA might have been solubilized and dislocated during fractionation is discussed and not excluded. In fact, the ease with which ECA is released from the bacterial cell and its high surface activity (coating of erythrocytes, lymphocytes, latex particles) might have led to contamination of some fractions. Thus, the hitherto described observations, while showing clearly that ECA is a constituent of the outer membrane, do not allow one to state definitely that this is its only cellular location.

Investigation for ECA presence in glycine-induced spheroplasts of *Salmonella typhi* revealed rather low ECA activity by hemagglutination and hemagglutination inhibition as well as by immunization. However, only the entire spheroplast suspension was active and not the supernate fraction or the lysed suspension (*Whang* and *Neter*, 1965b). Similar results were obtained recently in collaboration with *Gmeiner* using labile spheroplasts from *Proteus mirabilis* in comparison to the wild types. The spheroplasts contained some residual outer membrane material which contained ECA as demonstrated by ferritin-labeling (in ultrathin sections) and fluorescent anti-ECA antibodies. The cytoplasmic membrane was not labeled with either marker, indicating absence of ECA. Consequently, stable protoplast L forms of *P. mirabilis* without detectable outer membrane did not show ECA in supernates of heated cultures, nor were they stainable with ferritin-antibody conjugates against ECA (*Rinno* et al., 1978; *Männel* et al., 1977). These observations indicate that ECA is absent from the cytoplasmic membrane, and that if it is present in the cytoplasm then this is only in trace amounts.

In view of the localization of ECA in the outer membrane, the question arises whether ECA, like LPS, is an important virulence factor. It was possible to construct nearly isogenic strains of *Salmonella* differing only in regard to ECA, and these strains were studied to evaluate the possible role of ECA as a virulence factor. When tested in i.p. infection of mice, the *S. typhimurium* strains containing ECA were more virulent than their ECA-sister strains. Al-

though the difference was not more than tenfold, it was statistically highly significant (*Valtonen* et al., 1976). It was further shown that absence of ECA did not affect the growth rate. The in vivo clearance revealed no differences between ECA-positive and ECA-negative sister strains, suggesting that phagocytosis is not the discriminating mechanism.

VII. Immunogenicity and Antigenicity of ECA

A. Studies with Free ECA

As discussed in the following paragraph, the LPS-linked ECA of R strains with R1 and R4 core types is immunogenic, regardless whether the immunization is carried out with heat-killed bacterial suspensions or with isolated (ECA-containing) LPS.

Under certain conditions, however, isolated free ECA of nonimmunogenic strains can be a potent immunogen too. The immunogenicity of isolated ECA was especially studied by *Neter* and his co-workers. First, the antibody response of rabbits to soluble and cell-attached ECA—obtained from crude supernates of heat-killed suspensions of *E. coli, Salmonella,* and *Shigella*—was investigated. Intravenous immunization of rabbits with soluble ECA resulted in either a minimal ECA response or none at all (*Neter* et al., 1964). Immunization with ECA attached in vitro to either erythrocytes (autologous, isologous, or heterologous), lymphocytes from patients with leukemia (*Whang* et al., 1965b), or mouse fibroplast (L) cells (*Neter* et al., 1964) resulted in a good ECA immune response. It was discussed that haptenic ECA of enteric bacteria other than *E. coli* 014 (and other immunogenic R mutants) may be rendered fully antigenic by attachment to cells. Crude supernates of these enteric bacteria also elicited ECA antibodies in the rabbit upon injection into the footpads of rabbits with or without Freund's adjuvant (*Gorzynski* et al., 1963). Therefore it was a surprise that ECA of nonimmunogenic strains proved highly immunogenic in the rabbit upon i.v. injection when separated from the simultaneously present O antigen by means of 85% ethanol (*Suzuki* et al., 1964a). Immunization of rabbits with mixtures of ethanol-soluble and ethanol-insoluble (LPS) fractions resulted in a minimal immune response to ECA, showing that it is the simultaneous presence of LPS which leads to a decrease in ECA immunogenicity (*Suzuki* et al., 1964c). When ECA (ethanol-soluble fraction) and LPS (ethanol-insoluble fraction) were mixed in ethanol the decrease in immunogenicity was larger than with aqueous mixtures. No decrease was observed in immunogenicity when both fractions were administered separately in different ear veins (*Suzuki* et al., 1964c). Lipid A preparations injected together with ethanol-soluble ECA also interfere with the antibody response of rabbits (*Whang* et al., 1965a; *Whang* and *Neter*, 1967b). In all these experiments, in which no circulating antibodies were found, immunologic priming of the rabbits occurred, since animals responded with a rapid and more intense production of ECA antibodies upon the injection of subeffective doses of isolated ECA (*Neter* et al., 1966; *Neter*, 1969). *Neter* designated this new type of unresponsiveness as antigen-

associated immunosuppression (*Whang* and *Neter*, 1969) in order to point out that an intimate contact between ECA and the inhibitory substances is required for such an inhibition. Many different lipids and detergents were found to have similar effects on ECA immunogenicity. Lipid constituents (e.g., cardiolipin and cholesterol) of higher animals were included in the study. Cardiolipin interfered with the immune response to ECA (*Whang* and *Neter*, 1968 b) and also to another bacterial antigen, the Rantz-type common antigen of gram-positive bacteria (*Rantz* et al., 1956), but did not affect the antibody response to O antigen. In contrast to the finding with cardiolipin, cholesterol markedly enhanced the immune response to ECA (*Whang* and *Neter*, 1968 b). Assuming that ECA present in supernates of bacteria is complexed with LPS and that this complex interferes with ECA immunogenicity, then cholesterol can be expected to interact with this complex formation. Separate administration of supernate fraction and cholesterol does, indeed, not enhance the antibody response to ethanol-soluble ECA.

Since some serum components are known to complex with bacterial antigens (*Gorzynski* and *Neter*, 1966; *Praino* and *Neter*, 1977) it was investigated whether normal serum has an influence on ECA immunogenicity. It was reported (*Whang* and *Neter*, 1969) that serum almost completely prevented the immune response of ECA, provided antigen and serum were incubated prior to immunization. Even autologous serum obtained from the animal before the immunization proved to be immunosuppressive. Normal serum does not bind or destroy the antigenic determinant as judged from the unchanged antibody neutralization capacity of ECA in the presence or absence of serum. Investigations of isolated alpha-one protein shows that it is one or *the* substance in serum that accounts for inhibiting the production of circulating antibodies (*Whang* and *Neter*, 1969).

Other known immunosuppressants are chlorphenesin (3-*p*-chloro-phenoxy-1,2-propanediol) (*Whang* et al., 1970), gangliosides, Triton X-100, Tween 20, and methyl palmitate (*Agarwal* and *Neter*, 1971). Also, membranes of *Mycoplasma arthritidis* (*Bergquist* et al., 1974) show immunosuppression of ECA and antigens of gram-positive bacteria. However, glycerol triacetate, sphingomyelin, carotenes, cerebrosides, and cephalin do not alter the immune response of ECA in the rabbit (*Agarwal* and *Neter*, 1971).

It was postulated that ECA and the inhibitor interact to form a complex that affects early events of the immune response such as antigen uptake or processing. Most of the hitherto known immunosuppressants are membrane active compounds—lipids or ampholytes—which can readily complex with other lipids or amphipathic substances such as ECA. By this interaction they may alter the molecular size of ECA aggregates and thus subsequently the immunogenicity.

High-speed centrifugation of ethanol-soluble ECA resulted in the separation of aggregated and soluble fractions: Quantitation of ECA determinant showed that supernate and pellet neutralized ECA antibodies to almost the same extent. Immunization with both fractions, however, showed that only the pellet fraction was immunogenic. Nonetheless, also the animals that obtained a single injection of the supernate material were immunologically primed, since they responded more rapidly and with a considerably enhanced production of ECA antibodies

on the booster injection (*Whang* et al., 1970). Millipore filtration of ethanol-soluble ECA showed the same results: The aggregated fraction removed by filtration was immunogenic and the antigen in the filtrate used in comparable amounts was largely nonimmunogenic. The effect of repeated freezing and thawing on the immunogenicity of the filtrate was then investigated. Millipore-filtered ECA was rendered immunogenic by repeated freezing and thawing, presumably by reaggregation. Heating for 1 h at 100° C reduced the immunogenicity of ECA by more than 90% (*Whang* et al., 1971a; *Neter* et al., 1973b), and freezing and thawing of heated antigen restored immunogenicity to a significant degree, especially when repeated cycles of freezing and thawing were made. It is therefore clear that the ethanol-soluble ECA consists of two forms, one nonaggregated and nonimmunogenic, the other aggregated and immunogenic. These aggregation forms are in equilibrium; the equilibrium can be influenced by changes in temperature (heating and freezing) and by adding surface active components (see above, antigen-associated immunosuppressants).

It is evident that in most cases the small size of nonaggregated ECA is responsible for the observed poor or absent immunogenicity. Molecular weight determinations have been carried out with isolated ECA (*Männel* and *Mayer*, 1978a) and a molecular weight of 2700 was found for the portion readily dissolvable in 85% ethanol (about ten *N*-acetyl amino sugar units).

However, not all observations with antigen-associated immunosuppressants can be explained by decreasing the molecular weight of ECA by disaggregation (*Neter*, 1971). Lipopolysaccharides isolated from ECA-negative strains were found to be unable to produce the immunosuppression of the ECA immune response in rabbits in contrast to similar preparations obtained from ECA-positive strains (*Whang* et al., 1976). The common characteristic of the nonactive strains is a defect in the *rfe* gene(s) that is responsible for synthesis of ECA and certain *Salmonella* O chains. Whether other hitherto unrecognized functions are determined by *rfe*, which make LPS of rfe^+ strains immunosuppressive, is not known. The question whether immunologic tolerance is involved in this unresponsiveness has been previously discussed (*Mäkelä* and *Mayer*, 1976). It has to be pointed out, however, that true tolerance was not observed, since animals responded with an enhanced and accelerated antibody formation after a secondary immunization. This shows that the animals were primed and not tolerant (*Neter*, 1971).

Studies on ECA immunogenicity were preferentially performed in rabbits; unimmunized rabbits only very rarely have ECA titers, and rabbits respond very well to the i.v. injection of cell-bound or isolated ECA. In rabbits, transplacental transfer of ECA antibodies to fetuses has been demonstrated following immunization of pregnant rabbits and demonstration of (19S) antibodies to ECA in pooled sera of fetuses (*Whang* et al., 1967).

In addition to rabbits, the immune response of ECA was also investigated in several strains of mice, in guinea-pig and in man. Several mouse strains (C57BL/6Ha, DBA/2Jax, CBA/St, and Swiss albino) were found to differ significantly in their capacity to produce ECA antibodies, regardless whether immunization was carried out with or without Freund's adjuvant (*Gorzynski* et al., 1970). Ethanol-soluble fractions of heated supernatants of *E. coli* 014 and 0111

were used and injected i.p. Although titers were generally moderate or low, CBA/St and C57BL/6Ha responded better than DBA/2Jax or an outbred strain of Swiss albino mice.

In contrast to the results obtained with ethanol-soluble ECA, *McCabe* and *Greely* (1973) obtained high ECA titers in CF1 mice with heat-killed suspensions of *E. coli* 014, administered i.p. (three consecutive days for 2 weeks and then once a week for another 2 weeks). The final titers were in the range of 640–2560 and antisera were reported to contain about equal amounts of 7S and 19S antibodies. Similar results were obtained recently (*Männel* and *Mayer*, 1978b) using NMRI mice and i.v. injection of heat-killed *E. coli* 014 cells. Immunization of additional NMRI mice using isolated, highly purified ECA of *Salmonella montevideo* yielded only transient formation of antibodies on day 7 after the first injection. After this response the titers fell off rapidly and were not enhanced by a booster injection 3 weeks later. However, when the same preparation of purified ECA was coated on acetic acid-treated bacterial cells (Re mutant *S. minnesota* R595, ECA-negative) and injected i.v. or i.p. into NMRI mice, high ECA-specific titers were obtained. The specificity of the immune response was ascertained by serum adsorptions.

Hartley albino guinea-pigs were immunized by *Morgenstern* and *Gorzynski* (1973) with ECA extracted from heated bacterial suspensions (*E. coli* 014 and 0111) by means of 85% ethanol. ECA was administered with complete Freund's adjuvant into the four footpads of the animals. Nine days after the initial immunization, serum samples were taken for determination of the hemagglutinin titers and the animals were tested for delayed hypersensitivity. A s.c. booster injection was given on day 20. ECA elicited a humoral antibody response, although the titers were low, presumably because of the selected immunization schedule and the route of administration. A cellular immune response was also indicated by significant differences in the dermal reactivity between immunized and control animals. It was pointed out that soluble ECA is not completely free of endotoxic LPS (see next paragraph), and that endotoxin is highly active in cutaneous reactions. Thus, the reactions observed might be due to LPS contaminations.

B. Studies with LPS-Linked ECA

Kunin (1963) related the peculiar ability of *E. coli* 014 to induce antibody formation against ECA to the relatively large amount of ECA in this species. It was later shown (*Neter* et al., 1964; *Suzuki* et al., 1966) that i.v. injection of equivalent amounts of ECA from *E. coli* 014 and from other enterobacterial strains caused a specific immune response in the rabbit only with the former antigen. The difference in ECA immunogenicity of enterobacterial strains is therefore due to qualitative and not to quantitative differences. Fractionation of supernates of heated cultures by 85% aqueous ethanol usually separates ECA (ethanol-soluble) and LPS (insoluble in ethanol) (*Suzuki* et al., 1964a), showing that normally these antigens are independent antigenic entities. However, attempts to separate ECA and LPS of the ECA-immunogenic strain *E. coli* 014:K7 by these techniques were unsuccessful. This strain produces ethanol-soluble

and ethanol-insoluble ECA, both demonstrable by means of hemagglutination and hemagglutination inhibition (*Suzuki* et al., 1966). Studies on the immunogenicity of these two fractions revealed that it is only the insoluble ECA which is responsible for ECA antibody formation in the rabbit, thus showing that the immunogenicity of *E. coli* 014 is due to its content of this fraction of ethanol-insoluble ECA (*Suzuki* et al., 1966).

A deeper understanding of the chemical nature of this peculiar type of immunogenic ECA came from the study of a number of R mutants of *E. coli* 08 and 09 and of *Shigella sonnei* and *Sh. boydii*, which proved to be immunogenic for ECA. These mutants were serologically characterized as belonging to the R1 core type (*Schmidt* et al., 1969; *Mayer* and *Schmidt*, 1971; *Whang* et al., 1972b; *Mayer* and *Schmidt,* 1973) and as being partly cross-reacting with LPS of *E. coli* 014. It was then revealed (*Schmidt* et al., 1974) that acapsular mutants of *E. coli* 014:K7 were always rough (R) and of an additional core type (R4). This observation gave strong support to the assumption that ECA immunogenicity and R character are always related. A study of the criteria which R mutants must fulfill to be immunogenic for ECA revealed that the R core must be complete (*Mayer* and *Schmidt*, 1971; *Marx* and *Petcovici*, 1976) and of the R1 or R4 type. The presence of O-specific chains as well as incomplete synthesis of the cores results in loss of the ECA immunogenicity. Nonimmunogenic R mutants of *E. coli* and *Shigella* having the correct and complete R cores were characterized by genetic recombination studies as being deficient in *rfa*L or *rfe* genes. The latter mutants have a defect in ECA as well as in O-chain synthesis. Although mutants with a defective *rfa*L gene are able to synthesize O chains, they are nevertheless of R character since the defect in the translocase system (*rfa*L gene product) prevents the transfer of the O chains to the receptor R core (*Stocker* and *Mäkelä*, 1971). By analogy it is assumed that the defect in the *rfa*L gene prevents the attachment of ECA to the R1 or R4 core (*Schmidt* et al., 1976a). The finding of *rfa*L defects in nonimmunogenic R1/R4 type mutants is a strong indication of an enzyme-dependent transfer of ECA onto the R cores.

It has been pointed out recently (*Galanos* et al., 1977) that it is not a rare event for haptens in the bacterial cell to replace O chains of LPS to some extent and under certain conditions. Examples of such haptens are the T1 and T2 antigens of *Salmonella* and acidic polysaccharides of *E. coli* serotypes. The transfer of the T chains to the core likewise requires an active translocase, i.e., the product of the *rfa*L gene (*Sarvas* et al., 1967; *Stocker* and *Mäkelä*, 1971).

Lipopolysaccharides extracted from R mutants by the PCP extraction procedure (*Galanos* et al., 1969) are usually completely free of ECA as evidenced by serological techniques (*Mayer* and *Schmidt*, 1971), but this does not apply to the ECA-immunogenic R strains. ECA and R specificity in these mutants are, to some extent at least, closely associated. ECA follows R LPSs during all purification steps: it is precipitated with LPS during ultracentrifugation, it coats erythrocytes under the same conditions (heat or alkali treatment) as LPS. From these observation it was concluded that ECA and R LPS are covalently linked (*Mayer* et al., 1972).

In a recent study (*Kiss* et al., 1978) the linkage of ECA with the R1 type LPS was investigated further, making use of a set of three genetically related R mutants of *E. coli* 08:K27$^-$ (the *rfb*$^-$ mutant F470, the *rfa*L$^-$ mutant F614, the ECA-negative rfe$^-$ mutant F1283). Only the LPS of F470 and to a lesser extent the degraded (R1) polysaccharide of F470 were active in inhibiting an ECA hemagglutinating system. None of the polysaccharide or oligosaccharide fractions of the other strains were inhibitory. As it is known from previous work on the chemistry of the haptenic form of ECA that it is mainly composed of *N*-acetyl-D-glucosamine and *N*-acetyl-D-mannosaminuronic acid (*Männel* and *Mayer*, 1978a, b) and that it can be labeled by growth of the cells in the presence of (^{14}C)-*N*-acetyl-D-glucosamine (*Kiss*, 1975; *Kiss* et al., 1978), an attempt was made to correlate the presence of these sugars and inhibitory activity. The presence of labeled amino sugars, notably the rare constituent D-mannosaminuronic acid, agreed perfectly with the outcome of the inhibition experiments: only those fractions active in inhibiting the ECA system contained the sugars. This result shows that ECA is chemically linked to the R core region in R1 LPSs and that core-linked ECA can be cleaved off from lipid A by acetic acid treatment. It was further learned that the degree of the R core substitution is rather small, but apparently enough to render ECA immunogenic.

Immunization of rabbits with PCP-extracted LPS of the R1 type mutants produced ECA and R1 antibodies with the preparation from F470; exclusively R1 antibodies were formed by immunization with LPS from F614 and F1283 (*Rinno*, 1979).

In different strains of *E. coli* K-12 the complete core is partly substituted at the nonreducing end with *N*-acetylglucosamine or with another substitute, believed to be ManNAcUA. The attachment site is the 6-position of the nonreducing core-terminal glucose (*Prehm* et al., 1976). *E. coli* K-12 is partly associated with ECA immunogenicity (*Mäkelä* and *Mayer*, 1976), although the titers are much lower than those obtained with R1 or R4 mutants.

The detailed chemical structures of the R1 and the R4 cores was elucidated recently (*Feige* and *Stirm*, 1976; *Ørskov* et al., 1977; *Feige* et al., 1977). They are depicted in a simplified manner in Fig. 7. It is of interest that the two core types have in common a terminal tetrasaccharide chain which is substituted by β-linked D-glucose in the case of the R1 core and by β-linked D-galactose in the case of the R4 core. The structural similarity explains the serological cross-reaction between R1 and R4 type mutants (*Mayer* and *Schmidt*, 1971, 1973); the structural differences might be responsible for the differing phage pattern (*Mayer* and *Schmidt*, 1973) and the different reactivity with the lectin of *Ricinus communis* which is specific for terminal (β-linked) D-galactosyl units (*Mayer* and *Feige*, unpublished observations).

Immunization of rabbits with acetic acid-treated bacteria according to *Galanos* et al. (1971) shows that ECA immunogenicity is sensitive to acid treatment. ECA immunogenicity of R1 type mutants is almost completely lost after acetic acid treatment, whereas ECA immunogenicity of R4 mutants seems to be of higher acid resistance and is only partly lost upon this treatment. That this surprising result is not explicable by differences in the amount of LPS-associated

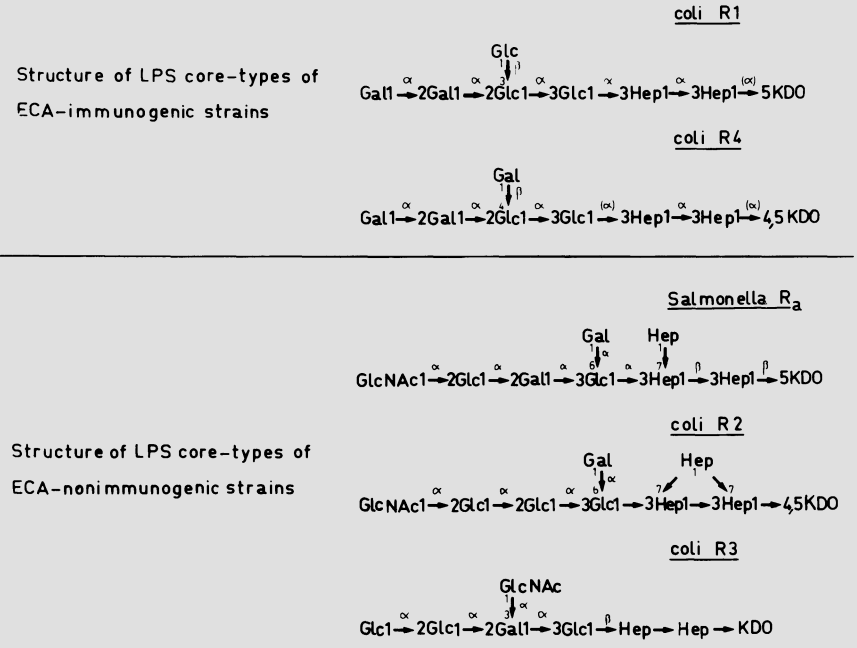

Fig. 7. Schematic structure of the LPS core type in ECA-immunogenic strains *E. coli* R1 and R4 (*Feige* and *Stirm*, 1976; *Ørskov* et al., 1977; *Feige* et al., 1977) and in ECA-nonimmunogenic strains *Salmonella* Ra (*Lüderitz* et al., 1971), *E. coli* R2 (*Hämmerling* et al., 1971) and *E. coli* R3 (*Johnston* et al., 1967 and *Jannson* et al., 1979)

ECA is indicated by examining the titers against lipid A in the same antisera by the passive hemolysis test. The respective titers were very high in R1 antisera and only of a medium height in R4 antisera. The reciprocal correlation between lipid A immunogenicity and ECA (and R) immunogenicity again leads to the conclusion that it is the R oligosaccharide and not the lipid A to which ECA is linked. Acetic acid splits the ketosidic linkage between R core and lipid A formed by KDO, thus abolishing in a parallel way both ECA and R immunogenicity and creating lipid A immunogenicity, which otherwise is completely cryptic (*Mayer* et al., 1978; *Rinno*, 1978).

VIII. Biologic Significance

Many members of the family Enterobacteriaceae are causative agents of several diseases in man and animals. Therefore the ECA, shared by almost all strains of Enterobacteriaceae, has created considerable interest as a potential means of diagnosis as well as of prophylaxis. In addition, the existing antigenic similarity between ECA and certain host animal tissues, which has recently been demonstrated (see below), may play a role in the etiology of some diseases.

A. ECA in Toxicity Tests

Some attempts have been made to establish whether ECA exerts toxic and pyrogenic effects like the endotoxic LPS. *Kunin* (1963) tested a DEAE cellulose-purified ECA preparation from *E. coli* 014 in rabbits. It produced fever, but failed to show lethal effects when given in amounts of 500 µg. In contrast, LPS was lethal in amounts of 25 µg.

Similarly, an ECA preparation isolated from extracts of *Salmonella typhi* by chromatography on Sephadex G-200 was pyrogenic in rabbits only after i.v. administration of 250 and 500 µg, but not at a dose of 100 µg (*Johns* et al., 1973). Other adverse effects were not observed. It is noteworthy, however, that both these preparations had lost some characteristic properties of ECA: they failed to sensitize red blood cells and to elicit antibodies in rabbits (*Kunin*, 1963; *Johns* et al., 1973).

For their toxicity test, *Kessel* et al. (1966) used an ethanol-soluble preparation (*Suzuki* et al., 1964a) obtained from *S. typhimurium*, which had retained typical traits of ECA, namely immunogenicity and RBC-sensitizing capacity. They found that the preparation was nonlethal for mice at an i.p. dose of 1000 µg. In other endotoxin assays the ethanol-soluble preparation was also 100–1000-fold less efficient than LPS; promotion of a Schwartzman-like reaction by epinephrine in rabbits, production of nonspecific resistance and cytotoxicity on monolayers of macrophages. Thus there is no indication that ECA exerts effects characteristic of endotoxins, and it is likely that pyrogenic and other toxic effects at comparably high doses of ECA may be due to contamination with small amounts of LPS.

B. Role of ECA Antibodies in Disease

Generally, in normal human sera ECA antibodies have been found in very low titers only (*Kunin*, 1962, 1963; *Kunin* and *Beard*, 1963). A response of low magnitude and frequency to ECA was usually observed in patients with acute urinary tract infections due to Enterobacteriaceae (*Andersen,* 1966; *Diaz* and *Neter*, 1968; *Vosti* et al., 1964; *Whang* and *Neter*, 1963a) or with enteritis caused by *E. coli* or *Salmonella* (*Diaz* and *Neter*, 1968; *Whang* and *Neter*, 1963a). Recently, *Whang* et al. (1972b) have shown that repeated i.v. injections of viable cells of enterobacterial smooth wild-type strains into rabbits elicit very low ECA antibody titers only, whereas the administration of viable rough (R) mutants results in high antibody titers. Since enterobacterial infections are generally provoked by smooth strains, it is not surprising that ECA antibodies in sera of such patients are present in low titers only. In contrast, however, with shigellosis, pyogenic peritonitis, and chronic but not acute urinary tract infections (UTIs), high anti-ECA antibody titers were frequently encountered (*Whang* and *Neter*, 1963a; *Diaz* and *Neter*, 1968; *Neter* et al., 1973a, *Neter* et al., 1974; *Thomas* et al., 1977). With regard to shigellosis it is well-known that rough mutants arise in high frequency from smooth parental strains. This might explain the elevated level of ECA antibodies in patients with shigellosis.

Significant high titers of ECA antibodies, which run up to 160 or more in 55% of the cases studied, were obtained in sera of children with pyogenic peritonitis (*Neter* et al., 1973a). This is probably due to a massive antigenic stimulus and/or the emergence of R mutants. It has also been discussed (*Gorzynski*, 1976) that the high antibody level in peritonitis might reflect an immunogenic influence of cross-reacting antigens in tissues which is de-repressed by this kind of infection. However, the precise mechanism leading to this increase in antibodies remains to be elucidated. It is interesting to note that also in patients with inflammatory bowel disease a high antibody response to *E. coli* 014 has been demonstrated (*Perlmann* et al., 1967). *Neter* and *Whang* (1972) reported that in one patient with peritonitis the unusual height of ECA antibodies (titer 640) could be correlated with repeated isolations of an *E. coli* R1 type strain from the peritoneal fluid. This is consistent with the finding that the injection of viable R mutants in contrast to smooth (S) strains resulted in high ECA antibody titers in rabbits (*Whang* et al., 1972b).

It is documented that in patients with peritonitis the ECA antibody titers can persist for up to 2 years (*Griffiths* et al., 1976). Also, a long persistence of significantly elevated ECA antibody titers was reported in cases of chronic pyelonephritis. Japanese workers have suggested that the determination of ECA antibodies is of some value in the diagnosis of this disease (*Saito*, 1967; *Kudo*, 1970). Similarly, *Thomsen* and *Hjort* (1977) claimed that the detection of ECA antibodies, even when occurring in low but significant titers, might be a better serological parameter of *E. coli* infections of the urinary tract than testing for O antibodies. Other investigators deny the value of ECA antibody determination in pyelonephritis (*Andersen*, 1966; *Ahlstedt* and *Holmgren*, 1975; *Budde* et al., 1978).

The role of ECA antibodies for the diagnosis of UTIs deserves further attention. The mere demonstration, however, of ECA antibodies by passive hemagglutination (IgM mostly) alone apparently does not allow definite conclusions.

Most cases of human chronic pyelonephritis are thought to be due to bacterial infections, although bacteria are often not demonstrable in the urine or renal tissue. Therefore, it has been claimed that residual bacterial antigens persisting in the renal tissue in the absence of viable bacteria might induce immunologic mechanisms which in turn result in chronic lesions of the kidney. The immunofluorescence technique with ECA antiserum offers an easy method of detecting enterobacterial antigens in tissue, even long after the disappearance of viable bacteria. By this technique *Aoki* et al. (1969) detected ECA in renal tissue in confirmed pyelonephritis and in six out of seven cases of chronic "abacterial pyelonephritis," in which bacteria could not be cultivated. Other workers, however, found ECA in kidney tissues in only one out of nine cases of chronic and in three out of five cases of acute pyelonephritis (*Schwartz* and *Cotran*, 1973). *Thomsen* and *Hjort* (1973) reported that in experimental hematogenous pyelonephritis induced by various strains of *E. coli* in rats, ECA could be demonstrated in the acute phase. In six cases of chronic disease, however, ECA could not be demonstrated, whereas type-specific antigens could. This result was confirmed by two other more detailed studies (*Thomsen*, 1974; *Thomsen* and

Hjort, 1975). Also, in a study of human chronic renal disease, *Thomsen* and *Olsen* (1977) did not find any support for the presence of amorphous bacterial antigen material in renal tissue. By immunofluorescence O antigen was found in whole bacteria and amorphously in macrophages, whereas ECA was detectable in whole bacteria only. Amorphous antigens were not observed outside phagocytizing cells. Thus it appears unlikely that the progression of renal lesions is due to persistent bacterial antigens (*Thomsen* and *Olsen*, 1977). In connection with this, however, a report of *Westenfelder* and *Galanos* (1974) is of some relevance: they could show that lipid A, the endotoxic determinant common to all Enterobacteriaceae, persisted in the kidney tissue after injection into renal pelvis of dogs and induced an abacterial nephritis. Moreover, formation of lipid A antibodies was observed. In another report (*Westenfelder* et al., 1977) it was demonstrated that in man a relation apparently exists between lipid A antibody titers and pyelonephritis.

Carrillo et al. (1966) and *Kumate* et al. (1971) studied the ECA content of *E. coli* strains isolated from feces obtained from healthy subjects and during episodes of diarrhea. *E. coli* strains with "high" content of ECA were found in stools of healthy newborns and after the diarrheal bout. Unexpectedly, ECA decreased significantly in *E. coli* strains isolated in cases of diarrhea. These findings are in some contrast to the report of *Valtonen* et al. (1976), which showed that ECA is a virulence factor (see Sect. VI).

C. Protective Activity

The identification of a common antigen in Enterobacteriaceae has opened the possibility to immunize with a single antigen against infections of different types of bacteria.

Domingue and *Neter* (1966a) showed that rabbit antibodies against ECA promote phagocytosis by polymorphonuclear leukocytes of *E. coli* and *Salmonella typhimurium* bacteria. *Pseudomonas aeruginosa*, which does not contain ECA, was not opsonized to a larger extent by ECA antiserum than by normal rabbit serum. A study of the bactericidal activities of ECA antibodies demonstrated that only cells of *E. coli* 014, and not those of *S. typhimurium*, were killed in the presence of complement. The recent finding (*Schmidt* et al., 1974) that *E. coli* 014 represents a rough strain may explain its sensitivity to complement action.

Intravenous immunization has been performed in human volunteers with ethanol-soluble ECA isolated from *E. coli* 0111 (*Gorzynski* et al., 1972b). The resulting ECA antibody titers ranged from 160 to 1280. The antisera of the immunized subjects showed higher opsonic activity than did the preimmunization sera (*van Oss* et al., 1972). Thus it was conceivable that ECA antibodies might play a role in protection against infections caused by ECA-containing bacteria. *Gorzynski* et al. (1971, 1972a) and *Gorzynski* and *Krasny* (1975c) vaccinated mice i.p. with *E. coli* 014 or with the ethanol-soluble ECA from *E. coli* 0111 and challenged with *S. typhimurium*. Both mouse strains, C57BL/Ha6 and Swiss albino mice, showed only transient but statistically significant protection.

Similarly, some prolongation of life after challenge with *S. typhimurium* was observed when mice were pretreated with horse red blood cells coated with ECA (*Gorzynski* and *Krasny*, 1975c). Passive immunization with rabbit ECA antiserum gave similar results (*Gorzynski* et al., 1971).

McCabe and *Greely* (1973) immunized CF1 mice i.p. with heat-killed *E. coli* 014 bacteria. The antibody titers increased from less than 20 to 640 or greater. Upon i.v. challenge with *Klebsiella pneumoniae* or *Proteus morganii* (first erroneously typed as *E. coli*; see *McCabe* et al., 1973b) the LD_{50} values were exactly the same in the immunized and in the control groups. Passive immunization with two rabbit ECA antisera gave no protection either, but a third serum did. This protective serum was shown to contain in addition to ECA antibodies another type of antibody directed against the Re LPS core determinant which is probably common to most Enterobacteriaceae (*McCabe* and *Greely*, 1973). Absorption of this serum with ECA did not abolish its protective capacity, whereas absorption with rough bacteria of the Re chemotype (*S. minnesota* R595) did.

These findings strongly support the notion that Re antigen may play a role as a cross-reactive protecting factor. This was also the conclusion of other studies with Re immunization in mice (*McCabe*, 1972). Similarly, it could be demonstrated that Re antibodies, but not ECA antibodies, have protective activity on human bacteremia caused by enteric bacteria (*McCabe* et al., 1972, 1973a; also see, however, *Greisman* et al., 1978).

Domingue et al. (1970) reported that i.v. vaccination of rabbits with heat-killed supernatants of *S. typhimurium* followed by ethanol-soluble ECA protected rabbits against experimental pyelonephritis after retrograde or hematogenous challenge with *Proteus mirabilis*. The protection was thought to be specific since vaccinated rabbits were not protected against *Pseudomonas aeruginosa*, which is not a member of the Enterobacteriaceae family and lacks ECA. Also, passive immunization with anti-ECA serum, but not with an absorbed serum free from ECA antibodies, led to protection against hematogenous challenge with *Proteus mirabilis*. Similar results were obtained when ethanol-soluble ECA was used alone for vaccination without priming injections of supernates of heat-killed suspensions (*Frentz* and *Domingue*, 1973; *McLaughlin* and *Domingue*, 1974). Thus the authors hoped to exclude a possible nonspecific action of endotoxin, which usually is a constituent in heat-treated extracts from gram-negative bacteria. Protection was not achieved against a capsulated *K. pneumoniae* strain which was also not opsonized by ECA antibodies; the heavy capsule probably masks the underlying ECA determinant.

The immunization experiments with ECA have been more promising in rabbits than in mice. It remains to be clarified whether this is due to the different kinds of infection or to the differences in animal species.

D. Serological Cross-Reactions Between Mammalian Tissues and ECA

After the demonstration of the so-called Forssman antigen by Rothacker, numerous antigenic similarities between tissues and bacteria have been detected,

of which a well-known example is the cross-reaction between heart tissue and streptococcal antigens (*Kaplan*, 1965). The cross-reactions between human tissues and gram-positive bacteria have been reviewed by *Zabriskie* (1967), the antigenic similarities between blood group substances and the Forssman antigen and bacterial substances by *Springer* (1971a, b). The significance of heterophile antigens for the host–parasite relationship and as a possible cause of pathogenicity was discussed by *Rowley* and *Jenkin* (1962) and *Jenkin* (1963).

Broberger and *Perlmann* (1959) first demonstrated antibodies against a colon antigen in sera of patients suffering from ulcerative colitis. Similar antigens were found to exist in both colon and feces of germ-free rats (*Perlmann* et al., 1967), and it was further shown that rats could be made autoimmune by injection of rabbit colon. The finding that sera of patients with ulcerative colitis and of animals made autoimmune by injection of germ-free colon have antibodies reactive with *E. coli* 014, the classical ECA-immunogenic strain, was a far-reaching discovery (*Perlmann* et al., 1965). Purified colon antigen is a heat-stable glycoprotein related to, but not identical with, blood group substances (*Carlsson* et al., 1978) which can be enriched by phenol–water extraction. Using sera of ulcerative colitis patients, hemagglutination of red blood cells coated with colon antigen extracted from germ-free animals can be inhibited by *E. coli* 014 LPS or by crude phenol–water extracts of *E. coli* 014 (*Lagercrantz* et al., 1968). That the inhibition of the cross-reaction is due to ECA rather than to the 014 ("R4," see above) LPS is indicated by a similar inhibition in tests in which *E. coli* 08 phenol–water extract was used for sensitization of erythrocytes and anti-*E. coli* 014 as antiserum. This system could also be inhibited by germ-free rat colon. The use of germ-free animals as a source of colon and feces precludes contamination with bacterial antigen which could interfere with the interpretation of the results.

Anticolon antibodies in ulcerative colitis may be formed as a result of tolerance breakage through cross-reactive antigens (ECA) by the intestinal microflora (*Perlmann* et al., 1967; *Lagercrantz* et al., 1968). Since this microbial flora, and hence also the cross-reacting antigen, is present in healthy individuals as well, additional factors are required to explain the induction of anti-colon autoimmunity in ulcerative colitis.

Thayer et al. (1969) confirmed that elevated titers of antibodies against germ-free rat colon and *E. coli* 014 are more common in patients with inflammatory bowel disease (ulcerative colitis as well as granulomatous disease) than in healthy controls or in patients with unrelated gastrointestinal diseases (see also *Lagercrantz* et al., 1968). Subsequent studies of *Holmgren* et al. (1971) have extended the described immunologic cross-reaction between human or rat colon and ECA. They found that immunization of rabbits with certain strains of *E. coli* gave rise to antibodies that precipitated with antigens of human kidneys. Conversely, rabbit antisera to human kidney precipitated antigenic material of the same *E. coli* strains. One of the strains included in the study was *E. coli* 014, the classical ECA-immunogenic strain. The existence and the localization of the *E. coli* cross-reactive antigens in normal human kidneys was verified and investigated by immunofluorescence studies. One of these cross-reacting antigens was 02 antigen, another was identified as ECA. The latter antigen cross-reacted

with the colon-derived antigen (*Holmgren* et al., 1972) as evidenced by gel precipitation. Absorption of the rabbit antisera against the human kidney with liver and human erythrocytes (AB, Rh^+) had no influence on the cross-reactivity of ECA and colon. This proves that blood group antigens, transplantation antigens, and special liver antigens are not related to the kidney antigen that shows the cross-reactivity (see, however, *Morgenstern* and *Gorzynski*, 1977).

The biologic significance of this cross-reaction remains to be clarified. In this respect it is of interest that kidney enlargement and tubular localization of antibody in mice immunized with cross-reactive *E. coli* bacteria have been found (*Holmgren* et al., 1975). The possibility that renal lesions and impaired kidney functions sometimes observed in patients with ulcerative colitis might be due to autoantibodies or lymphocytes with reactivity against colon and kidney antigen is discussed by *Holmgren* et al. (1972, 1975). Using the leukocyte migration test *Eckhardt* et al. (1975a, b) demonstrated that 71% of patients with ulcerative colitis showed cellular immunity against ECA, and 93%, cellular immunity against human fetal intestinal antigens. A high incidence of cellular immunity against ECA was also observed in patients with liver cirrhosis (64%), whereas patients with Crohn's disease had a rather low incidence (12%–26%). It is concluded that only a weak tolerance against intestinal antigens exists and that the high percentage of cellular immune reactions in patients with cirrhosis of the liver indicates an impairment of the physiological elimination of antigens by the liver (*Eckhardt* et al., 1975a, b; *Triger* et al., 1972). Similar studies by *Bull* and *Ignaczak* (1973) also showed that lymphocytes in inflammatory bowel disease showed an increased reactivity to ECA, indicating that ECA inducing cellular immunity might be operative in this disease.

Morgenstern and *Gorzynski* (1975, 1977) investigated sterile organs of human subjects free of infection for the presence of an antigen that cross-reacts with ECA. Homogenates of liver, kidney, fetal heart tissue, and muscle were heated and, together with the ethanol-soluble fractions, examined for their capacity to react with ECA antibodies (inhibition of an ECA hemaglutinating system) and to engender humoral or cellular events in rabbits or guinea pigs. Extracts of heated homogenates and ethanol-soluble fractions of liver and kidney, but not of heart and muscle, inhibited ECA hemagglutination. The same fractions also primed rabbits for rapid and specific ECA hemagglutinin response to a single administration of ECA and produced cutaneous delayed hypersensitivity in guinea pigs to ECA. Similar results were also obtained using aborted human fetuses, indicating that the cross-reactivity observed between human tissues and ECA is not attributable to indigenous bacteria present in the tissues. This was also demonstrated by sterility controls.

Gorzynski and *Krasny* (1975a, b) discovered similar cross-reacting antigens in mice, these antigens being active in inhibition of an ECA hemagglutinating system. C57BL/6Ha and Swiss albino strains, which had previously been examined (*Gorzynski* et al., 1970) for their capacity to produce ECA antibodies on i.p. immunization, were used. Although a cross-reactive antigen was identified in liver extracts of both mouse strains by neutralization of ECA hemagglutinins, only the liver fraction of Swiss albino strain elicited a significant number of rosette-forming cells in rabbit spleens. Studies of organs of gnotobiotic mice

showed a cross-reacting antigen in spleen, liver and kidney, but not in colon. It was suggested by *Gorzynski* and *Krasny* (1975b) that the presence of the cross-reactive material might be the reason for the poor response of mice to immunization with ECA and also for differences between mouse strains (*Gorzynski* et al., 1970). An antigen which cross-reacts with ECA has also been detected by inhibition of an ECA hemagglutinating system in extracts of rat tissues (livers, sera, and RBC) of several rat strains (*Krasny* and *Gorzynski*, 1977).

The importance of these cross-reactions, which are believed to be involved in the pathogenesis of certain autoimmune diseases, would make it very desirable to test whether ECA is solely responsible for these cross-reactions in all cases. The current availability of ECA negative strains of *E. coli* 014, which can be used, e.g., for absorption of anti 014 antiserum, should provide a definite answer to this question.

IX. Other "Common Antigens" in Gram-negative Bacteria

Recently, interest in common antigenic structures has increased because of the possible significance of these surface structures for questions of "natural immunity" and among other phenomena, for recognition processes. While it is not attempted here to cover the field of antigens shared by nonrelated gram-negative species extensively, it seems important to distinguish ECA from other common antigens. Some of the data on cross-reacting antigens may also concern ECA, even when the relationship is not obvious.

Brodhage (1961, 1962a and b) found that urea extracts of several *Salmonella* serotypes contain an antigen (C antigen, *c*ommon antigen) that modifies SRBC for hemagglutination with antisera prepared against urea extracts from otherwise serologically unrelated species. Some commercial antisera were also reactive with urea extract, e.g., Vi antisera and *Shigella sonnei* test antisera, as were sera from patients suffering from enterobacterial infections (Widal-positive sera). The independence of C antigen from *Salmonella* R antigens (*Brodhage*, 1962b) was documented. The antigen could also be extracted using a variety of other hydrogen bond-splitting reagents (thiourea, guanidine, potassium rhodanide etc.). The data reported for C antigen make it very likely that *Brodhage* was dealing with the same antigen discovered and described 1 year later by *Kunin* et al. (1962) as common enterobacterial antigen. Investigations carried out very recently with precipitating monospecific ECA antisera showed that urea extracts of Enterobacteriaceae indeed contain ECA (*Kuhn* and *Mayer*, unpublished). The observed reactivity of SRBC coated with urea extracts with a *Sh. sonnei* test serum is understandable from the fact that *Sh. sonnei* phase II has ECA in its immunogenic form (Mayer and Schmidt, 1971), and knowing that the S–R mutation occurs very readily with *Sh. sonnei*.

Well-characterized polymeric substances of the outer membrane of the gram-negative cell envelopes are O antigens (LPSs) and proteins. The isolation of the former is easily performed by phenol–water extraction (*Westphal* et al., 1952); the number of proteins isolated under undenaturing conditions and in a preparative scale is, however, comparatively small.

Lipopolysaccharides of Enterobacteriaceae and many other gram-negative families are composed of three different structural regions which are also under separate genetic control (*Lüderitz* et al., 1971; *Stocker* and *Mäkelä*, 1971): the O-specific chains, the core (R) oligosaccharide, and lipid A. The O chains, built up from repeating units of oligosaccharide, vary greatly in composition and structure and are responsible for the large number of serotypes existing, e.g., in *Salmonella* (*Kauffmann*, 1966; *Lüderitz* et al., 1971).

The O chains are linked to a core oligosaccharide with serological R specificity (R core), which in turn is bound via KDO units to the third structural entity, the lipid A. R mutants lacking O-specific chains can arise spontaneously. R antigens are also intermediates in O-antigen biosynthesis (*Robbins* and *Wright*, 1971).

Lipid A has—with a few notable exceptions (*Lüderitz* et al., 1978)—a very similar or even identical structure in many gram-negative species (*Hase* and *Rietschel*, 1976) and is therefore a strong candidate for a common antigen. Lipid A, however, anchors LPS in the outer membrane and is therefore usually embedded in it and not accessible for antibody-forming cells. Isolated lipid A is immunogenic, but lipid A antibodies react to a small extent, or not at all, with whole bacteria or S LPSs (*Galanos* et al., 1971). Nevertheless, using the passive hemolysis test, lipid A antibodies could be demonstrated with high frequency in sera of patients suffering from UTIs (*Westenfelder* et al., 1977), but not in sera from newborns and rarely in sera from healthy adults.

The R core region of Enterobacteriaceae shows more variability, although all *Salmonella* species seem to share the same R core type (*Lüderitz* et al., 1971). In *E. coli*, however, five different R core types have been described (R1–R4 and K-12) (*Ørskov* et al., 1977); some of them are also found among *Shigella* species (*Mayer* et al., 1973). In addition to the basal sugars, *Proteus mirabilis* contains in its R core D-galacturonic acid and therefore shows no cross-reaction with the core types of *E. coli* (*Kotelko* et al., 1977). R antibodies are often found in antisera prepared against S forms (*Schlecht* et al., 1971). For *Salmonella* it was shown that these are mainly directed against the higher chemotypes, Ra, Rb, and Rc. Enhanced antibody titers—especially against these three higher chemotypes—were encountered in sera from patients suffering from *Salmonella* infections (*Schlecht* et al., 1976). No significant rise in antibody titers against deeper R core regions (chemotypes Rd–Re) were found, although the deep R core region is the structure best preserved during evolution and therefore a prime candidate for a common antigen. Re type (heptose-less) LPSs are immunogenic (*Lüderitz* et al., 1966; *Lüderitz* et al., 1971), but the resulting Re antibodies are not capable of agglutinating S forms. Conjugated to fluorescein isothiocyanate, however, they can detect this common structure in heterologous bacteria (*Young* et al., 1975). *Eskenazy* et al. (1977) showed that isothiocyanate-conjugated antisera to *Salmonella* Re mutants showed a much stronger reaction when acid-hydrolyzed bacteria were used, indicating that hydrolysis uncovers shared glycolipid determinants responsible for cross-reactivity. Using this technique S forms of *Salmonella, Pseudomonas* and *Bordetella pertussis* proved reactive with Re antibodies.

In some contrast to the findings of *Schlecht* et al. (1976), *McCabe* et al. (1972) and *McCabe* (1976) found anti-Re antibodies in normal human sera and elevated titers in sera of patients with gram-negative infections. It was shown that Re antibodies are protective, in contrast to antibodies to other R chemotypes and to ECA. However, recent work in mice on the protective capacity of Re antibodies in passive transfer experiments showed that protection is not due to Re antibodies, since preimmunization sera showed the same degree of protection (*Ng* et al., 1976; *Greisman* et al., 1978).

Proteins of the outer membrane of enterobacterial cell envelopes have been studied in recent years by a number of groups (*Schnaitman*, 1973; *Hindenach* and *Henning*, 1975; *Rosenbusch*, 1974; *Lugtenberg* et al., 1975; for a review see *Braun* and *Hantke*, 1974), especially since many of them seem to be widely distributed and have interesting functions as receptors or structural elements (transmembrane channels, hydrophilic pores). Only some of them have been isolated under nondenaturing conditions without changes in their antigenic determinants. The (murein-) lipoprotein of *E. coli* is well characterized and is extremely resistant to denaturing conditions (*Braun*, 1975). It seems to be widely distributed in gram-negative bacteria (*Mayer* et al., 1973), although some enterobacterial species like *Proteus mirabilis* contain a structurally different lipoprotein (*Gmeiner* et al., 1978). In rabbit antisera prepared against whole bacteria (*E. coli, Salmonella, Shigella,* and even *Rhodopseudomonas gelatinosa*) antibodies against the *E. coli* B lipoprotein were present (*Braun* et al., 1976; *Drews* et al., 1978). The titers were lowest when encapsulated S forms were used for immunization and highest when deep rough mutants were used. Correspondingly, absorption of lipoprotein antibodies was most pronounced with deep rough mutants (*Braun* et al., 1976). In wild-type cells, lipoprotein is more or less buried in the outer membrane; its exposure is related to defects in that membrane. It was shown (*Smith*, 1977) that a humoral and local intrarenal antibody response to muropeptide-containing lipoprotein was obtained in rabbits with experimental hematogenous pyelonephritis. Striking differences in the lipoprotein antibody response were found in patients with various enterobacterial infections (*Griffiths* et al., 1977). Lipoprotein antibodies could not be detected in sera of healthy children and were present in only a small percentage of healthy adults, whereas 42% of patients with bacteremia due to enterobacterial infections produced these antibodies. The antibody response to lipoprotein did not correlate with the immune response to ECA (see above; *Neter* et al., 1973a; *Griffiths* et al., 1977).

By extracting the heptose-less mutant R595 of *Salmonella minnesota* with EDTA/MeOH (*Geyer*, 1977; *Geyer* and *Westphal*, 1977) a very basic protein (isoelectric point 10.3) was released which showed a characteristic association with the lipid A component of LPSs. This protein was therefore designated as "*k*omplexierendes" protein (k protein). It was obtained in a pure state by ion-exchange chromatography and affinity chromatography using k protein-specific antibodies linked to activated Sepharose 4B. Absorption of sera with heterologous strains of various enterobacterial genera showed that it represents a common surface antigen accessible for antibodies in both S and R forms. Whether k protein is engaged in anchoring lipid A in the outer membrane

is not known. Since the association with LPS is due to ionic interaction, it seems possible that also other charged macromolecules are interacting with this basic protein; ECA would be a candidate. *Marx* and *Petcovici* (1975) showed that ECA precipitated with lysozyme, which has an isoelectric point similar to the k protein.

Other proteins of the outer membrane are separable by SDS polyacrylamide gel electrophoresis. Some of them are widely distributed and dominate in their amounts ("major proteins"). The 34k, 35k, and 36k proteins described by *Nikaido* et al. (1977) and *Nurminen* (1978) form pores across the outer membrane through which hydrophilic compounds can diffuse. The resistance of these "porines" towards proteases can be exploited to attain the latter's isolation. The availability of mutants containing all except one of these porines allows the isolation of individueal porines (*Nurminen*, 1978), which can then be used as immunogens.

An anodically moving thermolabile antigen (ATA) was identified by *Seltmann* (1971) in a large number of gram-negative strains (Enterobacteriaceae, but also *Pseudomonas*). Affinity chromatography was used to obtain it in a serologically active form (*Seltmann* and *Reissbrodt*, 1976). It was characterized as a galactose-containing glycoprotein (*Reissbrodt* et al., 1976b). From the observed reduction of the conjugation frequency of plasmids and the neutralization of sex-specific phages in the presence of ATA antibodies, it was assumed that ATA is the F pili target on the recipient cell surface (*Reissbrodt* et al., 1976a).

A number of common antigens were reported from *S. typhi* rough strain R2 (*Chermann* et al., 1967a, b). These heterologous antigens, designated as r1, r2, r3, and r4, are lipoproteins of relatively low molecular weight (except for r4, which is of higher mol. wt.) and seem to be common to all Enterobacteriaceae. They do not fix to erythrocytes, show feeble immunogenicity and very low toxicity. It has been demonstrated that none of these antigens are identical to ECA (*Chermann* et al., 1967a).

A hydrophobic protein P_{PLP} isolated from the cell envelope of *Hydrogenomonas* (*Pseudomonas*) *facilis* was isolated and characterized by *Rittenhouse* et al. (1971). Antibodies to this protein agglutinate *H. facilis* cells, but also a variety of taxonomically unrelated gram-negative bacteria (and even some gram-positive microorganisms). Other bacteria did not agglutinate, but showed a precipitation line in immunoelectrophoresis with extracts of cell homogenates. The authors assume that in these strains P_{PLP}-related proteins were present in regions of intact cells that are not accessible to antibodies (*Rittenhouse* et al., 1973a). The surface location in *H. facilis* was documented by iodination with lactoperoxidase and by the use of ferritin-conjugated antibodies (*Rittenhouse* et al., 1973b).

Many other common antigens exist as revealed by screening gram-negative bacteria using serological techniques (*Kajser*, 1975). One antigen showed a high electrophoretic mobility and was found in all investigated *E. coli* strains, but also in *Proteus* and *Pseudomonas*. Antibodies against it were recognized, e.g., in antimeningococcal antisera. It was shown to contain some carbohydrate and to be heat labile. It seems similar to or identical with the ATA of *Seltmann* (1971). The heat lability and its presence in *Pseudomonas* show that it is unrelated to ECA.

Høiby (1975) used immunoelectrophoretic techniques (crossed immunoelectrophoresis) for a study of cross-reaction between *Pseudomonas aeruginosa* and 36 other bacterial species. Several cross-reacting antigens could also be found with *Bordetella pertussis* (*Høiby* et al., 1976). The nature or the functions of these cross-reacting antigens have not yet been elucidated.

X. ECA as Member of a Group of Aminuronic Acid-Containing Bacterial Surface Antigens

The identification of D-mannosaminuronic acid as one of the main constituents of ECA (*Männel*, 1976; *Männel* and *Mayer*, 1978a) and as an important marker for the presence of this antigen (*Männel* et al., 1978) raises the question of the distribution of hexosaminuronic acids in other bacterial antigens.

Probably due to their more or less pronounced sensitivity toward acid hydrolysis (*Perkins*, 1963), hexosaminuronic acids have escaped detection until recently, although it seems that they are quite widely distributed as constituents of surface structures of gram-negative and gram-positive bacteria. Until the present time, aminuronic acids with D-*gluco*, D-*manno*-, D-, and L-*galacto*-, L-*altro*-, and *gulo*-configuration have been characterized. D-Glucosaminuronic acid has been detected as constituent of the d-specific polysaccharide of *Hemophilus influenzae* d (*Williamson* and *Zamenhof*, 1963), of the protective antigen of *Staphylococcus aureus* (*Hanessian* and *Haskell*, 1964), and of capsular polysaccharide of *Achromobacter georgipolitanum* (*Smith*, 1968), as well as of an extracellular polysaccharide of black yeast, NRRL Y-6272 (*Jeanes* et al., 1971). D-Galactosaminuronic acid is present in the Vi antigen of *Salmonella typhi* and some *Citrobacter* and *E. coli* serotypes. Its structure as a per-acetylated homopolymer of $\alpha(1\rightarrow4)$-linked D-galactosaminuronic acid has been established by *Heyns* and *Kissling* (1967). Galactosaminuronic acid was also found in O antigens of photosynthetic bacteria (cf. *Drews* et al., 1978), L-galactosaminuronic acid in the lipopolysaccharide of *Pseudomonas aeruginosa* (*Wilkinson*, 1977). L-Altrosaminuronic acid was recently detected as a constituent of *Shigella sonnei* phase I lipopolysaccharide (*Kontrohr*, 1977), and gulosaminuronic acid occurs in combination with D-mannosaminuronic acid in the K15 antigen of *Vibrio parahaemolyticus* (*Torii* et al., 1973) and in the cell wall of *Halococcus* sp. (*Reistad*, 1974).

Mannosaminuronic acid was first discovered by *Perkins* (1963) as a constituent of an acidic cell wall polysaccharide of *Micrococcus lysodeikticus*, where it forms together with D-glucose the so-called teichuronic acid (*Hase* and *Matsushima*, 1970, 1972). Recently it has been shown (*Rohr* et al., 1977; *Stark* et al., 1977) that in the initial reactions of teichuronic acid biosynthesis three lipid precursors are formed: GlcNAc-carrier lipid, ManNAcUA-GlcNAc-carrier lipid, and $(ManNAcUA)_2$-GlcNAc-carrier lipid. Undecaprenol monophosphate is the carrier lipid and the third intermediate in teichuronic acid biosynthesis $(ManNAcUA)_2$-GlcNAc-carrier lipid) then serves as acceptor for the sequential and alternating addition of glucose (from UDPG) and *N*-acetyl-D-mannosaminuronic acid (from UDPManNAcUA) (*Stark* et al., 1977). The lipid-linked inter-

mediates seem to have a structure that might be identical to the proposed repeating unit of ECA (*Männel* and *Mayer*, 1978a; *Lugowski* and *Romanowska*, 1978). Whether a serological relationship exists has not been investigated, but the completed teichuronic acid does not cross-react with ECA, nor do the *E. coli* K7 or K56 antigens (*Männel* and *Mayer*, 1978b) (see below).

ManNAcUA is also, together with D-glucose, a constituent of the K7 antigen of *E. coli* (*Mayer*, 1969; *Ichihara* et al., 1974) and of the serologically related K56 antigen (*Flemming*, 1972). The K7 antigen occurs in *E. coli* 07 and 014, the latter being the strain in which *Kunin* et al. (1962) discovered ECA and which is still the strain often used for preparing ECA antisera, since it contains the immunogenic form of ECA. Acapsular mutants of *E. coli* 07:K7 contain ECA, and an ECA-negative *rfe*$^-$ mutant of *E. coli* 014:K7 produces the K7 antigen (*Mayer* and *Schmidt*, unpublished), indicating that ECA and K7 are separate antigenic entities. Until the present it was not possible to obtain an *rff*$^-$ mutant of *E. coli* 014. Due to the impairment of the biosynthesis of UDPManNAcUA (*Lew* et al., 1978; see Sect. V.), such a mutant is assumed to have lost both ECA and K7 antigenic specificities. Due to the occurrence of ECA in all Enterobacteriaceae, the enzymes for the biosynthesis of UDPManNAcUA and for the transfer of the sugar must also be ubiquitous.

As well as in teichuronic acid ManNUA was also found, together with *N*-acetyl-D-fucosamine, in a new surface antigen observed in a T mutant of *Staphylococcus aureus* (*Wu* and *Park*, 1971).

The list of hexosaminuronic acids occurring in bacterial antigens is still growing; therefore while the mere identification of ManNUA is a strong indication of ECA presence, it should be supplemented by a serological examination.

X. Summary and Concluding Remarks

The results of chemical analyses have shown that ECA is an amino sugar polymer consisting of *N*-acetyl-D-glucosamine and *N*-acetyl-D-mannosaminuronic acid, partly esterified by acetic and palmitic acids. The investigations of several mutants blocked in ECA production have revealed a close connection between the biosynthesis of ECA and LPS. Thus the *rfe* gene cluster is involved in the synthesis of O chains of several *Salmonella* and *E. coli* serotypes, indicating a common step in the synthesis of the polymers. Moreover, it has been demonstrated that the *rfa*L gene product needed for O chain and T polysaccharide transfer onto the LPS core also participates in the translocation of ECA onto the LPS core in ECA immunogenic strains.

As shown, ECA, like LPS, is a constituent of the outer cell membrane, which is the primary target of the host defense mechanisms. Therefore it was conceivable that the absence of ECA might impair the virulence of pathogenic bacteria. In fact, *Valtonen* et al. (1977) could demonstrate that ECA-negative *S. typhimurium* mutants were less virulent than ECA-positive strains. In connection with this, it is interesting to note that the absence of certain outer membrane proteins does not affect the virulence of *S. typhimurium* (*Valtonen* et al., 1977).

It is still not entirely clear whether ECA is a protective immunogen against enterobacterial infections. Thus the degree of protection of mice against infection with *S. typhimurium* was only minimal after vaccination with ECA. More promising were those experiments which showed that the vaccination with ECA results in rather good protection against experimental pyelonephritis in rabbits.

On the other hand the finding that ECA shares antigenic determinants with human and animal tissue (Sect. VIII.D) is of some relevance for the potential application of ECA as a prophylactic vaccine. Thus it has been discussed that the stimulation of the production of autoantibodies by cross-reacting bacterial antigens like ECA may play an important role in the genesis of autoimmune diseases like ulcerative colitis (*Perlmann* et al., 1967). Further studies are indicated to elucidate the antigenic cross-reactivities existing between tissue antigens and their potential role in the etiology of autoimmune diseases.

Acknowledgments. We thank Drs. Erwin Neter, Buffalo; P. Helena Mäkelä, Helsinki; and Otto Lüderitz, Freiburg, for invaluable discussions and for critically reading the manuscript. We are grateful to Drs. Nikaido, Berkeley and Romanowska, Wrocław for permission to quote from their unpublished work.

References

Agarwal, M.K., Neter, E.: Effect of selected lipids and surfactants on immunogenicity of several bacterial antigens. J. Immunol. *107*, 1448–1456 (1971)

Ahlstedt, S., Holmgren, J.: Alteration of the antibody response to *Escherichia coli* O antigen in mice by prior exposure to various somatic antigens. Immunology *29*, 487–496 (1975)

Andersen, H.J.: Studies of urinary tract infection in infancy and childhood. VII. The relation of *E. coli* antibodies in pyelonephritis as measured by homologous and common (Kunin) antigens. J. Pediatr. *68*, 542–550 (1966)

Andersson, J., Melchers, F., Galanos, C., Lüderitz, O.: The mitogenic effect of lipopolysaccharides on bone-marrow-derived mouse lymphocytes. J. Exp. Med. *137*, 943–953 (1973)

Aoki, S., Merkel, M., McCabe, W.R.: Immunofluorescent demonstration of the common enterobacterial antigen. Proc. Soc. Exp. Biol. Med. *121*, 230–234 (1966)

Aoki, S., Merkel, M., Aoki, M., McCabe, W.R.: Immunofluorescent localization of bacterial antigen in pyelonephritis. I. The use of antisera against the common enterobacterial antigen in experimental renal lesions. J. Lab. Clin. Med. *70*, 204–212 (1967)

Aoki, S., Imamura, S., Aoki, M., McCabe, W.R.: "Abacterial" and bacterial pyelonephritis. Immunofluorescent localization of bacterial antigen. N. Engl. J. Med. *281*, 1375–1382 (1969)

Bader, R.-E.: Ein neuer gemeinsamer Faktor der Typen I bis V von *Pasteurella pseudotuberculosis.* Zentralbl. Bakteriol. [Orig. A] *221*, 327–333 (1972)

Bergquist, L.M., Lau, B.H.S., Winter, C.E.: Mycoplasma-associated immunosuppression: effect on hemagglutinin response to common antigen in rabbits. Infect. Immun. *9*, 410–415 (1974)

Braun, V.: Covalent lipoprotein from the outer membrane of *Escherichia coli.* Biochem. Biophys. Acta *415*, 335–377 (1975)

Braun, V., Hantke, K.: Biochemistry of bacterial cell envelope. Annu. Rev. Biochem. *43*, 89–121 (1974)

Braun, V., Bosch, V., Klumpp, E.R., Neff, I., Mayer, H., Schlecht, S.: Antigenic determinants of murein-lipoprotein and its exposure at the surface of *Enterbacteriaceae.* Eur. J. Biochem. *62*, 555–566 (1976)

Broberger, O., Perlmann, P.: Autoantibodies in ulcerative colitis. J. Exp. Med. *110*, 657–674 (1959)

Brodhage, H.: Harnstoff-Extrakte aus gramnegativen Bakterien in der indirekten Hämagglutinationsreaktion. I. Harnstoff-Extrakte aus *S. typhi, paratyphi B, cholerae suis* und *Shig. sonnei.* Z. Hyg. *148*, 94–104 (1961)

Brodhage, H.: Urea-treated gram-negative bacilli in the indirect hemagglutination reaction. Nature *193*, 501–502 (1962a)

Brodhage, H.: Harnstoff-Extrakte aus gramnegativen Bakterien in der indirekten Hämagglutinationsreaktion. II. Die serologische Beziehung zwischen dem „C-Antigen" und dem Rauh-Antigen. Z. Hyg. *148*, 208–213 (1962b)

Buchanan, R.E., Gibbons, N.E. (eds.): Bergey's manual of determinative bacteriology, 8th ed. Baltimore: Williams & Wilkins 1974

Budde, E., Nimmich, W., Naumann, G.: Die Bedeutung des Common-Antigens für die Serodiagnostik der chronischen Pyelonephritis. Dtsch. Gesundheitswesen *33*, 1–6 (1978)

Bull, D.M., Ignaczak, T.F.: Enterobacterial common antigen-induced lymphocyte reactivity in inflammatory bowel disease. Gastroenterology *64*, 43–50 (1973)

Carlsson, H.E., Sundblad, G., Hammarström, S., Perlmann, P., Gustafsson, B.E.: Immunological studies in ulcerative colitis. Extraction, fractionation, and immunological characterization of germ-free rat colon. Arch. Biochem. Biophys. *187*, 366–375 (1978)

Carrillo, J., Hashimoto, B., Kumate, J.: Content of heterogenetic antigen in *Escherichia coli* and its relationship to diarrhea in newborn infants. J. Infect. Dis. *116*, 285–296 (1966)

Chedid, L., Parant, M., Parant, F., Boyer, F.: A proposed mechanism for natural immunity to enterobacterial pathogens. J. Immunol. *100*, 292–301 (1968)

Chermann, J.C., Digeon, M., Raynaud, M.: Non-identité des antigènes hétérologues r1, r2, r3 et de l'antigène commun de Kunin. Ann. Inst. Pasteur Paris *112*, 375–398 (1967a)

Chermann, J.C., Raynaud, M., Digeon, M.: Etude des divers antigènes elaborés par *Salmonella typhi* rough (souche R_2). Ann. Inst. Pasteur Paris *112*, 375–398 (1967b)

Conley, F.E., Johns, M.A., McCabe, W.R.: Serological analysis of common enterobacterial antigen extracted by picric acid and ethanol methods. Am. Soc. Microbiol. Annu. Meeting Abstr. *B25*, p. 15 (1976)

Diaz, F., Neter, E.: Antibody response to the common enterobacterial antigen of children with shigellosis, salmonellosis or urinary tract infection. Am. J. Med. Sci. *256*, 18–24 (1968)

Diaz, F., Neter, E.: Production of common enterobacterial antigen by pigment-producing, gram-negative bacilli. J. Bacteriol. *97*, 453–454 (1969)

Domingue, G.J., Johnson, E.: Isolation of subcellular fractions containing immunogenic enterobacterial common antigen. Z. Immunitätsforsch. *148*, 23–38 (1974)

Domingue, G.J., Johnson, E.: The common antigen of enterobacteriaceae and its biological significance. In: The immune system and infectious diseases. 4th Convoc. Immunol., Buffalo, N.Y., 1974. *Neter, E., Milgrom, F.* (eds.), pp. 242–262. Basel: Karger 1975

Domingue, G.J., Neter, E.: Inhibition by lipopolysaccharide of immune phagocytosis of latex particles modified with common antigen of enteric bacteria. Proc. Soc. Exp. Biol. Med. *121*, 133–137 (1966a)

Domingue, G.J., Neter, E.: Opsonizing and bactericidal activity of antibodies against common antigen of *Enterobacteriaceae.* J. Bacteriol. *91*, 129–133 (1966b)

Domingue, G.J., Neter, E.: The plaque test for the demonstration of antibodies against the enterobacterial common antigen produced by lymph node cells. Immunology *13*, 539–545 (1967)

Domingue, G.J., Salhi, A., Rountree, C., Little, W.: Prevention of experimental hematogenous and retrograde pyelonephritis by antibodies against enterobacterial common antigen. Infect. Immun. *2*, 175–182 (1970)

Drews, G., Weckesser, J., Mayer, H.: Cell envelopes. In: The photosynthetic bacteria. *Clayton, R.K., Sistrom, W.R.* (eds.). New York: Plenum Press 1978

Eckhardt, R., Heinisch, M., Meyer zum Büschenfelde, K.H.: Cellular immune reactions against common antigen, small intestine, and colon antigen in patients with Crohn's disease, ulcerative colitis and cirrhosis of the liver. Scand. J. Gastroenterol. *11*, 49–54 (1975a)

Eckhardt, R., Heinisch, M., Meyer zum Büschenfelde, K.H.: Zelluläre Immunreaktionen gegenüber Common Antigen und humanen Dünn- bzw. Dickdarmantigenen bei Patienten mit Enteritis regionalis, Colitis ulcerosa und Leberzirrhose. Z. Gastroenterol. *13*, 413–417 (1975b)

Eskenazy, M., Konstantinov, G., Ivanova, R., Strahilov, D.: Detection by immunofluorescence of common antigenic determinants in unrelated gram-negative bacteria and their lipopolysaccharides. J. Infect. Dis. *135*, 965–969 (1977)

Eylar, E., Barber, C.: Common enterobacterial antibodies in anti-*Yersinia enterocolitica* sera. Microbios Letters *3*, 187–190 (1976)

Feige, U., Stirm, S.: On the structure of the *Escherichia coli* C cell wall lipopolysaccharide core and on its ϕX 174 receptor region. Biochem. Biophys. Res. Commun. *71*, 566–573 (1976)

Feige, U., Jann, B., Jann, K., Schmidt, G., Stirm, S.: On the primary structure of the *Escherichia coli* R 4 cell wall lipopolysaccharide core. Biochem. Biophys. Res. Commun. *79*, 88–95 (1977)

Flemming, H.C.: Vergleichende analytisch-chemische Untersuchungen an O- und K-Antigenen einiger typischer Vertreter der K-Antigen-Klasse B. Diploma-Work, University of Freiburg i.Br. 1972

Frentz, G., Domingue, G.: Effects of immunization with ethanol-soluble enterobacterial common antigen on in vivo bacterial clearance and hematogenous pyelonephritis. Proc. Soc. Exp. Biol. Med. *142*, 246–252 (1973)

Galanos, C., Lüderitz, O.: Electrodialysis of lipopolysaccharides and their conversion to uniform salt forms. Eur. J. Biochem. *54*, 603–610 (1975)

Galanos, C., Lüderitz, O., Westphal, O.: A new method for the extraction of R lipopolysaccharides. Eur. J. Biochem. *9*, 245–249 (1969)

Galanos, C., Lüderitz, O., Westphal, O.: Preparation and properties of antisera against the lipid-A component of bacterial lipopolysaccharides. Eur. J. Biochem. *24*, 116–122 (1971)

Galanos, C., Lüderitz, O., Rietschel, E.T., Westphal, O.: Newer aspects of the chemistry and biology of bacterial lipopolysaccharides, with special reference to their lipid A component. In: International Review of Biochemistry. Biochemistry of Lipids II, Vol. 14. *Goodwin, T.W.* (ed.), pp. 239–335. Baltimore: University Park Press 1977

Geyer, R.: Proteine der äußeren Membran Gram-negativer Bakterien. Isolierung und Charakterisierung eines Proteins aus Salmonella mit Affinität zu Lipopolysaccharid. Thesis, University of Freiburg i.Br. 1977

Geyer, R., Westphal, O.: Isolation and characterization of a lipopolysaccharide-binding protein from the outer membrane of *Salmonella minnesota* Re. Acta Microbiol. Acad. Sci. Hung. *24*, 168 (1977)

Gmeiner, J.: Glyceraldehyde phosphate at the reducing terminus of *Salmonella* O haptens. Eur. J. Biochem. *51*, 449–457 (1975)

Gmeiner, J., Kroll, H.-P., Martin, H.H.: The covalent rigid-layer lipoprotein in cell walls of *Proteus mirabilis*. Eur. J. Biochem. *83*, 227–233 (1978)

Gorzynski, E.A.: Biological significance of enterobacterial antigen (CA) and engendered antibodies. Milit. Med. *141*, 696–699 (1976)

Gorzynski, E.A., Krasny, S.A.: Cross-reactivity between organ extracts of gnotobiotic mice and enterobacterial common antigen. J. Reticuloendothel. Soc. *17*, 346–352 (1975a)

Gorzynski, E.A., Krasny, S.A.: Immunological mimicry between mouse tissue and enterobacterial common antigen. Immunol. Commun. *4*, 39–49 (1975b)

Gorzynski, E.A., Krasny, S.A.: Effect of erythrocytes treated with enterobacterial common antigen on experimental *Salmonella typhimurium* infection of mice. Med. Microbiol. Immunol. *161*, 163–170 (1975c)

Gorzynski, E.A., Neter, E.: Influence of polymyxin B and normal rabbit serum on erythrocyte modifying capacity of common enterobacterial antigen. J. Bacteriol. *91*, 2110–2111 (1966)

Gorzynski, E.A., Neter, E.: Production of common antigen by enteric bacteria grown in a synthetic culture medium. Infect. Immun. *2*, 767–771 (1970)

Gorzynski, E.A., Whang, H.Y., Suzuki, T., Neter, E.: Differences in antibody response of rabbit to enterobacterial antigen after intravenous and subcutaneous injection with adjuvant. Proc. Soc. Exp. Biol. Med. *114*, 700–704 (1963)

Gorzynski, E.A., Neter, E., Ambrus, J.L.: Differences in antibody responses of mouse strains to enterobacterial common antigen. Proc. Soc. Exp. Biol. Med. *134*, 776–779 (1970)

Gorzynski, E.A., Ambrus, J.L., Neter, E.: Effect of common enterobacterial antiserum on experimental *Salmonella typhimurium* infection of mice. Proc. Soc. Exp. Biol. Med. *137*, 1209–1212 (1971)

Gorzynski, E.A., Priore, R.L., Neter, E.: Effect of immunization with common enterobacterial antigen on experimental *Salmonella typhimurium* infection of mice. Immunol. Commun. *1*, 123–130 (1972a)

Gorzynski, E.A., van Oss, C.J., Ambrus, J.L.: The hemagglutinin response of human subjects to common enterobacterial antigen. Infect. Immun. *5*, 625–626 (1972b)

Greisman, S.E., Dubuy, J.B., Woodward, C.L.: Experimental gram-negative sepsis: Reevaluation of the ability of rough mutant antisera to protect mice. Proc. Soc. Exp. Biol. Med. *158*, 482–490 (1978)

Griffiths, E.K., Jewett, T.C., Neter, E.: Suppression by peritoneal irritants of the antibody response to common enterobacterial antigen. J. Reticuloendothel. Soc. *18*, 32b (1975)

Griffiths, E.K., Jewett, T.C., Neter, E.: Duration of antibody responses to common enterobacterial and O antigens of children with pyogenic peritonitis. Infection *4*, 1–4 (1976)

Griffiths, E.K., Yoonessi, S., Neter, E.: Antibody response to enterobacterial lipoprotein of patients with varied infections due to *Enterobacteriaceae*. Proc. Soc. Exp. Biol. Med. *154*, 246–249 (1977)

Hammarström, S., Perlmann, P.: Immunological studies of ulcerative colitis-immunochemistry of common antigen of *Enterobacteriaceae* (Kunin) -relation to lipopolysaccharide core structure. Acta Pathol. Microbiol. Scand. [B] *80*, 165–165 (1972)

Hammarström, S., Carlsson, H.E., Perlmann, P., Svensson, S.: Immunochemistry of the common antigen of *Enterobacteriaceae* (Kunin). J. Exp. Med. *134*, 565–576 (1971)

Hämmerling, G., Lüderitz, O., Westphal, O., Mäkelä, P.H.: Structural investigation on the core polysaccharide of *Escherichia coli* 0100. Eur. J. Biochem. *22*, 331–344 (1971)

Hanessian, S., Haskell, T.H.: Structural studies on Staphylococcal polysaccharide antigen. J. Biol. Chem. *239*, 2758–2662 (1964)

Hase, S., Matsushima, Y.: Structural studies on a glucose-containing polysaccharide obtained from *Micrococcus lysodeikticus* cell walls. I. Biochem. *68*, 723–730 (1970)

Hase, S., Matsushima, Y.: Structural studies on a glucose-containing polysaccharide from cell walls of *Micrococcus lysodeikticus*. III. Determination of the structure. Biochem. *72*, 1117–1128 (1972)

Hase, S., Rietschel, E.Th.: Isolation and analysis of the lipid A backbone. Lipid A structure of lipopolysaccharides from various bacterial groups. Eur. J. Biochem. *63*, 101–107 (1976)

Hayaishi, O., Kornberg, A.: Metabolism of phospholipides by bacterial enzymes. J. Biol. Chem. *206*, 647–663 (1954)

Heyns, K., Kiessling, G.: Strukturaufklärung des Vi-Antigens aus *Citrobacter freundii* (E. coli) 5396/38. Carbohyd. Res. *3*, 340–353 (1967)

Hindenach, I., Henning, U.: The major proteins of the outer cell envelope membrane. Preparative isolation of all major membrane proteins. Eur. J. Biochem. *59*, 207–213 (1975)

Høiby, N.: Cross-reactions between *Pseudomonas aeruginosa* and thirty-six other bacterial species. Scand. J. Immunol. *4*, Suppl. 2, 187–196 (1975)

Høiby, N., Hertz, J., Andersen, V.: Cross-reactions between *Bordetella pertussis* and twenty-eight other bacterial species. Acta Pathol. Microbiol. Scand. [B] *84*, 395–400 (1976)

Holmgren, J., Hanson, L.Å., Holm, S.E., Kaijser, B.: An antigenic relationship between kidney and certain *Escherichia coli* strains. Int. Arch. Allergy Appl. Immunol. *41*, 463–474 (1971)

Holmgren, J., Hammarström, S., Holm, S.E., Ahlmen, J., Attman, P.O., Jodal, U.: Antigenic

relationship between human kidney, colon and common antigen of *Enterobacteriaceae*. Int. Arch. Allergy Appl. Immunol. *43*, 89–97 (1972)

Holmgren, J., Goldblum, R.M., Blomberg, J., Hanson, L.Å., Hermodson, S., Holm, S., Kaijser, B.: Antigenic relationship between kidney and enteric bacteria. In: 4th International Convocation on Immunology, *Neter, E., Milgrom, F.* (eds.), Buffalo, N.Y., 1974, pp. 263–271. Basel: Karger 1975

Ichihara, N., Ishimoto, N., Ito, E.: Enzymatic incorporation of *N*-acetyl-*D*-mannosaminuronic acid and *D*-glucose into a polysaccharide of Escherichia coli 014:K7:H$^-$. FEBS Lett. *40*, 309–311 (1974)

Jann, K., Westphal, O.: Microbial polysaccharides. In: The antigens. *Sela, M.* (ed.), Vol. III, pp. 1–125. New York: Academic Press 1975

Jannson, P.E., Lindberg, B., Lindberg, A.A., Wollin, R.: Structural studies on the enterobacteriaceae type R3 core polysaccharide. Carbohydr. Res. *68*, 385–389 (1979)

Jeanes, A., Burton, K.A., Cadmus, M.C., Knutson, C.A., Rowin, G.L., Sandford, P.A.: Extracellular black yeast polysaccharide composed of N-acetyl glucosamine and N-acetyl glucosaminuronic acid. Nature [New Biol.] *233*, 259–260 (1971)

Jenkin, C.R.: Heterophile antigens and their significance in the host-parasite relationship. Adv. Immunol. *3*, 351–376 (1963)

Johns, M.A., Whiteside, R.E., Baker, E.E., McCabe, W.R.: Common enterobacterial antigen. I. Isolation and purification from *Salmonella typhosa* 0:901. J. Immunol. *110*, 781–790 (1973)

Johnson, E.J., Domingue, G.J., Klein, G.D.: Anatomical locus of the common enterobacterial antigen. Z. Immunitätsforsch. *152*, 15–26 (1976)

Johnston, J.H., Johnston, R.J., Simmons, D.A.R.: The immunochemistry of *Shigella flexneri* O-antigens. The biochemical basis of smooth to rough mutation. Biochem. J. *105*, 79–87 (1967)

Jousimies, H., Mäkelä, P.H.: Genetic analysis of *Salmonella minnesota* R mutants with defects in the biosynthesis of the lipopolysaccharide core. J. Bacteriol. *119*, 753–759 (1974)

Kaijser, B.: Immunological studies of an antigen common to many gram-negative bacteria with special reference to *E. coli*. Characterization and biological significance. Int. Arch. Allergy Appl. Immunol. *48*, 72–81 (1975)

Kaijser, B., Hagberg, S., Hanson, L.Å., Olling, S.: A search for *Escherichia coli* antigens in kidneys from children with urinary tract infection by means of immunofluorescence. Pediatr. Res. *8*, 935–940 (1974)

Kanegasaki, S., Jann, K.: Membrane reconstitution in the biosynthesis of the 09 antigen of *Escherichia coli*. Demonstration of butanol soluble and water soluble intermediates. Eur. J. Biochem., in press 1979

Kaplan, M.H.: Autoantibodies to heart and rheumatic fever: the induction of autoimmunity to heart by streptococcal antigen cross-reactive with heart. Ann. N.Y. Acad. Sci. *124*, 904–915 (1965)

Kauffmann, F.: The bacteriology of *Enterobacteriaceae*. Copenhagen: Munksgaard 1966

Kauffmann, F.: Classification of bacteria: A realistic scheme with special reference to the classification of *Salmonella*- and *Escherichia*-species. Copenhagen: Munksgaard 1975

Kawamura, T., Ichihara, N., Ishimoto, N., Ito, E.: Biosynthesis of uridine diphosphate *N*-acetyl-*D*-mannosaminuronic acid from uridine diphosphate *N*-acetyl-*D*-glucosamine in *Escherichia coli*: separation of enzyme responsible for epimerization and dehydrogenation. Biochem. Biophys. Res. Commun. *66*, 1506–1512 (1975)

Kessel, R.W.I., Neter, E., Braun, W.: Biological activities of the common antigen of *Enterobacteriaceae*. J. Bacteriol. *91*, 465–466 (1966)

Kiss, P.: Versuche zur Identifizierung und Charakterisierung unbekannter, Glucosaminhaltiger Makromoleküle der Zellwand von Salmonella, Thesis, University of Freiburg i.Br. 1975

Kiss, P., Rinno, J., Golecki, J., Schmidt, G., Mayer, H.: Enterobacterial common antigen. II. Studies on the immunogenic form. In: Functions of microbial membranes. Symposium Tübingen, Abstract 1977

Kiss, P., Rinno, J., Schmidt, G., Mayer, H.: Structural studies on the immunogenic form of the enterobacterial common antigen. Eur. J. Biochem. *88*, 211–218 (1978)

Kondo, R.: Immunological studies on chronic pyelonephritis. IV. On the enterobacterial common and O antibody response in patients and rabbits, especially on the production of 7S and 19S antibodies. Jpn. J. Nephrol. *12*, 365–382 (1970)

Kontrohr, T: The identification of 2-amino-2-deoxy-L-altruronic acid as a constituent of *Shigella sonnei* phase I lipopolysaccharide. Carbohydr. Res. *58*, 498–500 (1977)

Kotetko, K., Gromska, W., Papierz, M., Sidorzyk, Z., Krajewska, D., Szer, K.: Core region in *Proteus mirabilis* lipopolysaccharide. J. Hyg. Epidemiol. Microbiol. Immunol. (Praha) *21*, 271–281 (1977)

Krasny, S.A., Gorzynski, E.A.: Immunological cross-reaction between enterobacterial common antigen and rat tissue. ASM-Meeting-1977, Am. Soc. Microbiol. Annu. Meeting Abstr. *E51*, 89 (1977)

Kudo, K.: Simplification of the serological method for the diagnosis of chronic pyelonephritis. I. Estimation of enterobacterial common antibody by microhemagglutination test using ethanol separated common antigen. Fukushima J. Med. Sci. *20*, 173–180 (1970)

Kumate, J., Cravioto, J., Hashimoto, B., Vega, L., Carillo, J.: Content of common antigen of *Escherichia coli* and diarrhea of newborns and infants in a Mexican preindustrial community. Ann. N.Y. Acad. Sci. *176*, 350–359 (1971)

Kunin, C.M.: Distribution of antibodies against various non-enteropathic *E. coli* groups. II. Relation to age, sex, and breed. Arch. Intern. Med. *110*, 676–686 (1962)

Kunin, C.M.: Separation, characterization, and biological significance of a common antigen in *Enterobacteriaceae*. J. Exp. Med. *118*, 565–586 (1963)

Kunin, C.M., Beard, M.V.: Serological studies of O antigens of *Escherichia coli* by means of the hemagglutination test. J. Bacteriol. *85*, 541–548 (1963)

Kunin, C.M., Beard, M.V., Halmagyi, N.E.: Evidence for a common hapten associated with endotoxin fractions of *E. coli* and other enterobacteriaceae. Proc. Soc. Exp. Biol. Med. *111*, 160–166 (1962)

Lagercrantz, R., Hammarström, S., Perlmann, P., Gustafsson, B.E.: Immunological studies in ulcerative colitis. IV. Origin of auto-antibodies. J. Exp. Med. *128*, 1339–1352 (1968)

Le Minor, L., Chalon, A.-M., Vèron, M.: Recherches sur la présence de l'antigène commun des «Enterobacteriaceae» (antigène Kunin) chez les «Yersinia», «Levinea», «Aeromonas» et «Vibrio». Ann. Inst. Pasteur (Paris) *123*, 761–774 (1972)

Lew, H.C., Nikaido, H., Mäkelä, P.H.: The biosynthesis of uridine diphosphate *N*-acetylmannosaminuronic acid in *rff* mutants of *Salmonella typhimurium*. J. Bacteriol. *136*, 227–233 (1978)

Lüderitz, O., Galanos, C., Risse, H.J., Ruschmann, E., Schlecht, S., Schmidt, G., Schulte-Holthausen, H., Wheat, R., Westphal, O., Schlosshardt, J.: Structural Relationships of Salmonella O and R Antigens. Ann. N.Y. Acad. Sci. *133*, 349–374 (1966)

Lüderitz, O., Westphal, O., Staub, A.M., Nikaido, H.: Isolation and chemical and immunochemical characterization of bacterial lipopolysaccharides. In: Microbial toxins. *Weinbaum, G., Kadis, S., Ajl, S.J.* (eds.), Vol. IV, pp. 145–233. New York: Academic Press 1971

Lüderitz, O., Galanos, C., Lehmann, V., Nurminen, M., Rietschel, E.Th., Rosenfelder, G., Simon, M., Westphal, O.: Lipid A: Chemical structure and biological activity. J. Infect. Dis. *128*, Suppl., 17–29 (1973)

Lüderitz, O., Galanos, C., Lehmann, V., Mayer, H., Rietschel, E.Th., Weckesser, J.: Chemical structure and biological activities of Lipid A's from various bacterial families. Naturwissenschaften *65*, 578–585 (1978)

Ługowski, C., Romanowska, E.: Enterobacterial common antigen: Isolation from *Shigella sonnei*, purification and immunochemical characterization. Eur. J. Biochem. *91*, 89–97 (1978)

Lugtenberg, B., Meijers, J., Peters, R., Hoek, P.v.d., Alphen, L.v.: Electrophoretic resolution of the 'major outer membrane protein' of *Escherichia coli* K12 into four bands. FEBS Lett. *58*, 254–258 (1975)

Mäkelä, P.H., Mayer, H.: Participation of lipopolysaccharide genes in the determination of the enterobacterial common antigen: analysis in *Salmonella* groups B and C. J. Bacteriol. *119*, 765–770 (1974)

Mäkelä, P.H., Mayer, H.: Enterobacterial common antigen. Bacteriol. Rev. *40*, 591–632 (1976)

Mäkelä, P.H., Jahkola, M., Lüderitz, O.: A new gene cluster *rfe* concerned with the biosynthesis of Salmonella lipopolysaccharide. J. Gen. Microbiol. *60*, 91–106 (1970)

Mäkelä, P.H., Mayer, H., Whang, H.Y., Neter, E.: Participation of lipopolysaccharide genes in the determination of the common enterobacterial antigen: analysis of R mutants of *Salmonella minnesota*. J. Bacteriol. *119*, 760–764 (1974)

Mäkelä, P.H., Schmidt, G., Mayer, H., Nikaido, H., Whang, H.Y., Neter, E.: Enterobacterial common antigen in *rfb* deletion mutants of *Salmonella typhimurium*. J. Bacteriol. *127*, 1141–1149 (1976)

Maeland, J.A., Digranes, A.: Common enterobacterial antigen in *Yersinia enterocolitica*. Acta Pathol. Microbiol. Scand. [B] *83*, 382–386 (1975a)

Maeland, J.A., Digranes, A.: Human serum antibodies against heat-stable antigens from *Yersinia enterocolitica*. Acta Pathol. Microbiol. Scand. [B] *83*, 451–456 (1975b)

Männel, D.: Isolierung und Charakterisierung des Common Antigen der Enterobacteriaceae. Thesis, University Freiburg i.Br., 1976

Männel, D., Mayer, H.: Isolation and chemical characterization of the enterobacterial common antigen. Eur. J. Biochem. *86*, 361–370 (1978a)

Männel, D., Mayer, H.: Serological and immunological properties of isolated enterobacterial common antigen. Eur. J. Biochem. *86*, 371–379 (1978b)

Männel, D., Gmeiner, J., Mäkelä, P.H., Mayer, H.: Enterobacterial common antigen: Studies on the haptenic form. In: Functions of Microbial Membranes. Symposium Tübingen, Abstract 1977

Männel, D., Mäkelä, P.H., Mayer, H.: Enterobacterial common antigen in mutant strains of *Salmonella*. J. Bacteriol. *135*, 348–353 (1978)

Mancini, G., Carbonara, A.O., Heremans, J.F.: Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry *2*, 235–254 (1965)

Marx, A., Mayer, H.: The action of phospholipase A on the common enterobacteriaceae antigen of the Kunin-type. Zentralbl. Bakteriol. [Orig. A] *226*, 224–231 (1974)

Marx, A., Petcovici, M.: Immunochemical studies on purified common enterobacterial antigen. Zentralbl. Bakteriol. [Orig. A] *233*, 486–494 (1975)

Marx, A., Petcovici, M.: Role of the *rfa* locus in immunogenicity of common enterobacterial antigen (Kunin). Infect. Immun. *13*, 360–364 (1976)

Marx, A., Petcovici, M., Nacescu, N., Mayer, H., Schmidt, G.: Demonstration of enterobacterial common antigen by bacterial agglutination. Infect. Immun. *18*, 563–567 (1977)

Mayer, H.: D-Mannosaminuronsäure-Baustein des K7-Antigens von *Escherichia coli*. Eur. J. Biochem. *8*, 139–145 (1969)

Mayer, H., Schmidt, G.: Hämagglutinine gegen ein gemeinsames Enterobacteriaceen-Antigen in *E. coli* R1-Antiseren. Zentralbl. Bakteriol. [Orig. A] *216*, 299–313 (1971)

Mayer, H., Schmidt, G.: The occurrence of three different lipopolysaccharide cores in *Shigella* and their relationship to known enterobacterial core types. Zentralbl. Bakteriol. [Orig. A] *224*, 345–354 (1973)

Mayer, H., Schmidt, G., Whang, H.Y., Neter, E.: Biochemical basis of the immunogenicity of the common enterobacterial antigen. Infect. Immun. *6*, 540–544 (1972)

Mayer, H., Schlecht, S., Braun, V.: Immunogenicity of lipoprotein (murein-lipoprotein) of gram-negative bacteria. Joint Meeting of the European Societies for Immunology, Strasbourg, Abstract 1973

Mayer, H., Ruschmann, E., Rinno, J.: Differential effect of heat and acid-treatment on the immunogenicity of bacterial antigens: common antigen, lipoprotein, lipid A and R-antigen. Liblice Meeting, Abstract 1978

McCabe, W.R.: Immunization with R mutants of *S. minnesota*. I. Protection against challenge with heterologous gram-negative bacilli. J. Immunol. *108*, 601–610 (1972)

McCabe, W.R.: Immunoprophylaxis of gram-negative bacillary infections. Annu. Rev. Med. *27*, 335–341 (1976)

McCabe, W.R., Greely, A.: Common enterobacterial antigen. II. Effect of immunization on challenge with heterologous bacilli. Infect. Immun. *7*, 386–392 (1973)

McCabe, W.R., Kreger, B.E., Johns, M.: Type-specific and cross-reactive antibodies in Gram-negative Bacteremia. N. Engl. J. Med. *287*, 261–267 (1972)

McCabe, W.R., Johns, M., DiGenio, T.: Common enterobacterial antigen. III. Initial titers

and antibody response in bacteremia caused by gram-negative bacilli. Infect. Immun. *7*, 393–397 (1973a)

McCabe, W.R., Johns, M.A., Zinner, S.H., DiGenio, T., Greely, A.: Type specific and cross reactive antigens of Gram-negative bacilli in protection against infection. In: Gram-negative bacterial infections and mode of endotoxin action. *Urbaschek, B., Urbaschek, R., Neter, E.* (eds.), pp. 112–120. New York: Springer 1973b

McLaughlin, J.C., Domingue, G.J.: The immunologic role of the ethanol-soluble enterobacterial common antigen versus experimental renal infection. Immunol. Commun. *3*, 51–71 (1974)

Measel, J.W.: Type-specific antibody-response to enterobacterial common antigen. Microbios Lett. *4*, 139–145 (1977)

Measel, J.W.: Chemical studies on enterobacterial common antigen. J. Am. Osteopath. Soc. *77*, 398 (1978)

Measel, J.: Partial-purification and chemical identification of the enterobacterial common antigen by affinity chromatography. Microbios L. *7*, 19–24 (1978)

Melchers, F., Braun, V., Galanos, C.: The lipoprotein of the outer membrane of *Escherichia coli:* a B-lymphocyte mitogen. J. Exp. Med. *142*, 473–482 (1975)

Morgenstern, M.A., Gorzynski, E.: Immune response of guinea pigs to common enterobacterial antigen. Immunol. Commun. *2*, 495–506 (1973)

Morgenstern, M.A., Gorzynski, E.A.: Cross-reactivity between enterobacterial common antigen and human tissue. J. Reticuloendothel. Soc. *18*, 32 (1975)

Morgenstern, M.A., Gorzynski, E.A.: Immunogenic cross-reactivity between human tissues and the enterobacterial common antigen. Infect. Immun. *17*, 36–42 (1977)

Neter, E.: Bacterial hemagglutination and hemolysis. Bacteriol. Rev. *20*, 166–188 (1956)

Neter, E.: Indirect bacterial hemagglutination, and its application to the study of bacterial antigens and serologic diagnosis. Pathol. Microbiol. (Basel) *28*, 859–877 (1965)

Neter, E.: Endotoxins and the immune response. Curr. Top. Microbiol. Immunol. *47*, 82–124 (1969)

Neter, E.: Discussion-Suppression of immunogenicity of common enterobacterial antigen. Ann. N.Y. Acad. Sci. *181*, 151–152 (1971)

Neter, E., Whang, H.Y.: The common antigen of gram-negative bacteria. In: Cellular antigens. *Nowotny, A.* (ed.), pp. 14–21. Heidelberg, New York: Springer 1972

Neter, E., Bertram, L.F., Zak, D.A., Murdock, M.R., Arbesman, C.E.: Studies on hemagglutination and hemolysis by *Escherichia coli* antisera. J. Exp. Med. *96*, 1–15 (1952)

Neter, E., Whang, H.Y., Suzuki, T., Gorzynski, E.A.: Differences in antibody response of rabbit to intravenously injected soluble and cell-attached enterobacterial antigen. Immunology *7*, 657–664 (1964)

Neter, E., Whang, H.Y., Lüderitz, O., Westphal, O.: Immunological priming without production of circulating bacterial antibodies conditioned by endotoxin and its lipoid A component. Nature *212*, 420–421 (1966)

Neter, E., Kennedy, E.A., Jewett Jr., Th.C.: Antibody response to common enterobacterial antigen of children with pyogenic peritonitis. Infection *1*, 12–16 (1973a)

Neter, E., Whang, H.Y., Mayer, H.: Immunogenicity and antigenicity of endotoxic lipopolysaccharides: reversible effects of temperature on immunogenicity. J. Infect. Dis. *128*, 56–60 (1973b)

Neter, E., Merrin, C., Surgalla, M.J., Wajsman, Z.: Shigella sonnei bacteremia–Unusual antibody response from immunosuppressive therapy following renal transplantation. Urology *4*, 198–200 (1974)

Ng, A.-K., Chen, Ch.-L.O., Chang, Ch.-M., Nowotny, A.: Relation of structure to function in bacterial endotoxins: serologically cross-reactive components and their effect on protection of mice against some gram-negative infections. J. Gen. Microbiol. *94*, 107–116 (1976)

Nikaido, H., Levinthal, M., Nikaido, K., Nakane, K.: Extended deletions in the histidine-rough-B region of the Salmonella chromosome. Proc. Natl. Acad. Sci. USA *57*, 1825–1832 (1967)

Nikaido, H., Song, S.A., Shaltiel, L., Nurminen, M.: Outer membrane of Salmonella. XIV. Reduced transmembrane diffusion rates in porin-deficient mutants. Biochem. Biophys. Res. Commun. *76*, 324–330 (1977)

Nurminen, M.: A mild procedure to isolate the 34K, 35K and 36K porins of the outer membrane of *Salmonella typhimurium*. FEMS Microbiology Letters *3*, 331–334 (1978)

Onuma, H.: An experimental study on the antigenicity of enterobacterial common antigen in rabbits. Especially on the adjuvant action of renal tissue. Jpn. J. Nephrol. *10*, 375–388 (1968)

Ørskov, I., Ørskov, F., Jann, B., Jann, K.: Serology, chemistry and genetics of O and K antigens of *Escherichia coli*. Bacteriol. Rev. *41*, 667–710 (1977)

Osborn, M.J., Rick, P.D., Lehmann, V., Rupprecht, E.: Structure and biogenesis of the cell envelope of gram-negative bacteria. Ann. N.Y. Acad. Sci. *235*, 52–65 (1974)

Perkins, H.R.: A polymer containing glucose and aminohexuronic acid isolated from cell walls of *Micrococcus lysodeikticus*. Biochem. J. *86*, 475–483 (1963)

Perlmann, P., Hammarström, S., Lagercrantz, R., Gustafsson, B.E.: Antigen from colon of germfree rats and antibodies in human ulcerative colitis. Ann. N.Y. Acad. Sci. *124*, 377–394 (1965)

Perlmann, P., Hammarström, S., Lagercrantz, R., Campbell, D.: Autoantibodies to colon in rats and human ulcerative colitis: cross reactivity with *Escherichia coli* 014 antigen. Proc. Soc. Exp. Biol. Med. *125*, 975–980 (1967)

Praino, M., Neter, E.: Effect of serum from various animal species on erythrocyte attachment of endotoxins and other bacterial antigens. Infect. Immun. *18*, 612–616 (1977)

Prehm, P., Schmidt, G., Jann, B., Jann, K.: The cell wall lipopolysaccharide of *Escherichia coli* K-12. Structure and acceptor site for O-antigen and other substituents. Eur. J. Biochem. *70*, 171–177 (1976)

Rabin, B.S., Rogers, S.: Pathologic changes in the liver and kidney produced by immunization with intestinal antigens. Am. J. Pathol. *84*, 201–220 (1976)

Rantz, L.A., Randall, E., Zuckerman, A.: Hemolysis and hemagglutination by normal and immune serums of erythrocytes treated with nonspecific bacterial substance. J. Infect. Dis. *98*, 211–222 (1956)

Reissbrodt, R., Tschäpe, H., Seltmann, G.: The anodically-moving thermolabile antigen (ATA) of gram-negative bacteria: its function as the receptor for F pili. Z. Allg. Mikrobiol. *16*, 39–47 (1976a)

Reissbrodt, R., Kemmer, G., Seltmann, G.: Das anodisch wandernde thermolabile Antigen (ATA) von gramnegativen Bakterien: Seine molekulare Heterogenität und seine chemische Zusammensetzung. Z. Allg. Mikrobiol. *16*, 385–394 (1976b)

Reistad, R.: 2-Amino-2-deoxyguluronic acid: a constituent of the cell wall of *Halococcus* sp., strain 24. Carbohydr. Res. *36*, 420–423 (1974)

Rinno, J.: Enterobacterial Common Antigen. Lokalisation in der Bakterienzelle und immunogene Eigenschaften. Thesis, Universität Gießen 1979

Rinno, J., Golecki, J., Schmidt, G., Mayer, H.: On the localisation of the enterobacterial common antigen. Liblice Meeting, Abstract 1978

Rittenhouse, H.G., Heptinstall, J., McFadden, B.A.: A hydrophobic protein from the cell envelope of *Hydrogenomonas facilis*. Biochemistry *10*, 4045–4049 (1971)

Rittenhouse, H.G., Rodda, J.B., McFadden, B.A.: Immunologic cross-reacting proteins in cell walls of many bacteria. J. Bacteriol. *113*, 1400–1403 (1973a)

Rittenhouse, H.G., McFadden, B.A., Shumway, L.K., Heptinstall, J.: Cell surface protein of *Pseudomonas (Hydrogenomonas) facilis*. J. Bacteriol. *115*, 330–340 (1973b)

Robbins, P.W., Wright, A.: Biosyntheis of O-antigens. In: Microbial toxins. *Weinbaum, G., Kadis, S., Ajl, J.* (eds.), Vol. IV, pp. 351–368. New York: Academic Press 1971

Rohr, T.E., Levy, G.N., Stark, N.J., Anderson, J.S.: Initial reactions in the biosynthesis of teichuronic acid of *Micrococcus lysodeikticus* cell walls. J. Biol. Chem. *252*, 3460–3465 (1977)

Rosenbusch, J.P.: Characterization of the major envelope proteins from *Escherichia coli*. Regular arrangement on the peptidoglycan and unusual dodecyl sulfate binding. J. Biol. Chem. *249*, 8019–8029 (1974)

Rowley, D., Jenkin, C.R.: Antigenic cross-reaction between host and parasite as a possible cause of pathogenicity. Nature *193*, 151–154 (1962)

Rudczynski, A.B., Jackson, R.W.: The properties of a lipoteichoic acid antigen from Streptococcus pyogenes. Immunochemistry *15*, 83–91 (1978)

Saito, I.: Serological study of chronic pyelonephritis. Especially on the diagnostic value of the estimation of enterobacterial common antigen response. Fukushima J. Med. Sci. *14*, 45–53 (1967)

Sanderson, K.E., Hartman, P.E.: Linkage map of *Salmonella typhimurium*. Edition V. Microbiol. Rev. *42*, 471–519 (1978)

Sarvas, M., Lüderitz, O., Westphal, O.: Immunochemical studies on T1, S hybrids of *Salmonella paratyphi* B. Ann. Med. Exp. Fenn. *45*, 117–126 (1967)

Schlecht, S., Böhlck, I., Westphal, O.: Nachweis von Antikörpern gegen Salmonella-R-Antigen in Salmonella-O-Antiseren. Zentralbl. Bakteriol. [Orig.] *216*, 471–482 (1971)

Schlecht, S., Böhlck, I., Kuhn, M.: Nachweis von Antikörpern gegen Salmonella-R-Antigene bei Salmonellose-Kranken. Zentralbl. Bakteriol. [Orig. A] *236*, 48–66 (1976)

Schmidt, G., Jann, B., Jann, K.: Immunochemistry of R lipopolysaccharides of *Escherichia coli*. Different core regions in the lipopolysaccharides of group 08. Eur. J. Biochem. *10*, 501–510 (1969)

Schmidt, G., Jann, B., Jann, K.: Genetic and immunochemical studies on *Escherichia coli* 014:K7:H^-. Eur. J. Biochem. *42*, 303–309 (1974)

Schmidt, G., Männel, D., Mayer, H., Whang, H.Y., Neter, E.: The role of a lipopolysaccharide gene for immunogenicity of the enterobacterial common antigen. J. Bacteriol. *126*, 579–586 (1976a)

Schmidt, G., Mayer, H., Mäkelä, P.H.: Presence of *rfe* genes in *Escherichia coli:* their participation in biosynthesis of O antigen and enterobacterial common antigen. J. Bacteriol. *127*, 755–762 (1976b)

Schnaitman, C.A.: Outer membrane proteins of *Escherichia coli*. II. Heterogeneity of major membrane polypeptides. Arch. Biochem. Biophys. *157*, 553–560 (1973)

Schwartz, M.M., Cotran, R.S.: Common enterobacterial antigen in human chronic pyelonephritis and interstitial nephritis. N. Engl. J. Med. *289*, 830–835 (1973)

Seltmann, G.: Untersuchungen zur Antigenstruktur von Shigellen. IV. Isolierung und Reinigung eines thermolabilen Antigens von *Sh. sonnei*. Zentralbl. Bakteriol. [Orig. A] *219*, 324–335 (1971)

Seltmann, G., Reissbrodt, R.: Reinigung eines thermolabilen, in gramnegativen Bakterien vorkommenden Antigens (ATA) mit Hilfe der Affinitätschromatographie. Z. Allg. Mikrobiol. *14*, 237–239 (1976)

Smith, E.J.: Purification and properties of an acidic polysaccharide isolated from *Achromobacter georgipolitanum*. J. Biol. Chem. *243*, 5139–5144 (1968)

Smith, G.: Microbial surfaces in relation to pathogenicity. Bacteriol. Rev. *41*, 475–500 (1977)

Smith, J.W.: Local immune response to lipoprotein of the outer membrane of *Escherichia coli* in experimental pyelonephritis. Infect. Immun. *17*, 366–370 (1977)

Springer, G.F.: Blood-groups and Forssman antigenic determinants shared between microbs and mammalian cells. Prog. Allergy *15*, 9–77 (1971a)

Springer, G.F.: The importance of bloodgroup and Forssman antigenic determinants in interactions between man and microbes. In: Microbial toxins. *Kadis, S., Weinbaum, G., Ajl, S.J.* (eds.), Vol. 5, pp. 39–90. New York: Academic Press 1971b

Springer, G.F., Huprikar, S.V., Neter, E.: Specific inhibition of endotoxin coating of red cells by a human erythrocyte membrane component. Infect. Immun. *1*, 98–108 (1970)

Springer, G.F., Adye, J.C., Bezkorovainy, A., Jirgensons, B.: Nature and specificity of human erythrocyte membrane receptor for bacterial endotoxin. In: 4th International Convocation on Immunology, *Neter, E., Milgrom, F.* (eds.), Buffalo, N.Y., 1974, pp. 171–181. Basel: Karger 1975

Stark, N.J., Levy, G.N., Rohr, T.E., Anderson, J.S.: Reactions of second stage of biosynthesis of teichuronic acid of *Micrococcus lysodeikticus* cell walls. J. Biol. Chem. *252*, 3466–3472 (1977)

Starr, M.P., Mandel, M.: DNA base composition and taxonomy of phytopathogenic and other Enterobacteria. J. Gen. Microbiol. *56*, 113–123 (1969)

Stocker, B.A.D., Mäkelä, P.H.: Genetic aspects of biosynthesis and structure of *Salmonella*

lipopolysaccharide. In: Microbial toxins. *Weinbaum, G., Kadis, S., Ajl, S.J.* (eds.), Vol. V, pp. 369–438. New York: Academic Press 1971

Suzuki, T., Gorzynski, E.A., Neter, E.: Separation by ethanol of common and somatic antigens of *Enterobacteriaceae*. J. Bacteriol. *88*, 1240–1243 (1964a)

Suzuki, T., Gorzynski, E.A., Whang, H.Y., Neter, E.: Hemolysis in immune rabbits of autologous erythrocytes modified with common enterobacterial antigen. Experientia *20*, 75–76 (1964b)

Suzuki, T., Whang, H.Y., Gorzynski, E.A., Neter, E.: Inhibition by lipopolysaccharide (endotoxin) of antibody response of rabbit to common antigen of enterobacteriaceae. Proc. Soc. Exp. Biol. Med. *117*, 785–789 (1964c)

Suzuki, T., Whang, H.Y., Neter, E.: Studies of common antigen of enterobacteriaceae with particular reference to *Escherichia coli* 014. Ann. Immunol. Hung. *9*, 283–292 (1966)

Thayer, W.R., Brown, M., Sangree, M.H., Katz, J., Hersh, T.: Escherichia coli 0:14 and colon hemagglutinating antibodies in inflammatory bowel disease. Gastroenterology *57*, 311–318 (1969)

Thomas, V., Sanford, B., Carson, S., Forland, M., Shelokov, A.: Immune response in urinary tract infection determined by radioimmunoassay. Serum antibody levels against infecting bacterium and Enterobacteriaceae common antigen. Clin. Res. *25*, A 588–588 (1977)

Thomsen, O.F.: Localization and persistance of common enterobacterial antigen and type specific bacterial antigen in experimental pyelonephritis. Acta Pathol. Microbiol. Scand. [A] *82*, 277–286 (1974)

Thomsen, O.F., Hjort, T.: Immunofluorescent demonstration of bacterial antigen in experimental pyelonephritis with antiserum against common enterobacterial antigen. Acta Pathol. Microbiol. Scand. [A] *81*, 474–482 (1973)

Thomsen, O.F., Hjort, T.: Different rates of disappearence of common enterobacterial antigen and type-specific *E. coli* O-antigen in rat peritoneal macrophages. Acta Pathol. Microbiol. Scand. [C] *83*, 203–209 (1975)

Thomsen, O.F., Hjort, T.: Antibodies against *E. coli* O-antigen and common enterobacterial antigen in kidney-transplant recipients. Acta Pathol. Microbiol. Scand [B] *85*, 455–461 (1977)

Thomsen, O.F., Olsen, T.S.: Bacteria and bacterial antigen in the kidney in human chronic renal disease. Acta Pathol. Microbiol. Scand. [B] *85*, 440–448 (1977)

Torii, M., Sakakibara, K., Kuroda, K.: Occurrence of 2-amino-2-deoxyhexuronic acids as constituents of *Vibrio parahaemolyticus* K15 antigen. Eur. J. Biochem. *37*, 401–405 (1973)

Triger, D.R., Alp, M.H., Wright, R.: Bacterial and dietary antibodies in liver disease. Lancet *1972/I*, 60–63

Valtonen, M.V., Larinkari, U.M., Plosila, M., Valtonen, V.V., Mäkelä, P.H.: Effect of enterobacterial common antigen on mouse virulence of *Salmonella typhimurium*. Infect. Immun. *13*, 1601–1605 (1976)

Valtonen, M.V., Nurminen, M., Johansson, V., Mäkelä, P.H.: Mouse virulence of *S. typhimurium* mutants deficient in two major outer membrane proteins. Infect. Immun. *18*, 454–458 (1977)

van Oss, C.J., Ambrus, J.L., Gorzynski, E.A., Neter, E.: The opsonin response of human subjects to common enterobacterial antigen. Immunol. Commun. *1*, 69–75 (1972)

Vosti, K.L., Monto, A.S., Older, J.J., Rantz, L.A.: The serologic specificity of crude and purified antigen extracts of *Escherichia coli* in hemagglutination reactions with rabbit and human antisera. J. Immunol. *93*, 199–204 (1964)

Weinbaum, G., Kadis, S., Ajl, S.J. (eds.): Microbial Toxins, Vol. IV, V. New York, London: Academic Press 1971

Westenfelder, M., Galanos, C.: Experimental lipid A-induced nephritis in the dog, a possible role of lipid A in the pathogenesis of abacterial chronic pyelonephritis. Infection *2*, 174–177 (1974)

Westenfelder, M., Galanos, C., Withöft, A., Lang, G.: Vorkommen, Bedeutung und klinische Konsequenz der Lipoid A-Antikörper-Titer bei Patienten mit Harnwegsinfektionen. Infection *5*, 144–148 (1977)

Westphal, O., Lüderitz, O., Bister, F.: Über die Extraktion von Bakterien mit Phenol/Wasser. Z. Naturforsch. *7b*, 148–155 (1952)

Whang, H.Y., Neter, E.: Immunochemical studies of a heterogenetic enterobacterial antigen (Kunin). J. Bacteriol. *84*, 1245–1250 (1962)

Whang, H.Y., Neter, E.: Study of heterogenetic (Kunin) antibodies in serum of healthy subjects and children with enteric and urinary tract infections. J. Pediatr. *63*, 412–419 (1963a)

Whang, H.Y., Neter, E.: Comparative study of latex agglutination and hemagglutination tests for demonstration of enterobacterial antibodies. Am. J. Clin. Pathol. *40*, 136–142 (1963b)

Whang, H.Y., Neter, E.: Selective destruction by *Pseudomonas aeruginosa* of common antigen of enterobacteriaceae. J. Bacteriol. *88*, 1244–1248 (1964)

Whang, H.Y., Neter, E.: Destruction of common antigen of *Enterobacteriaceae* by a psychrophilic *Pseudomonas*. J. Bacteriol. *89*, 1436–1437 (1965a)

Whang, H.Y., Neter, E.: Demonstration of common antigen in *Salmonella typhi* spheroplasts. Can. J. Microbiol. *11*, 598–601 (1965b)

Whang, H.Y., Neter, E.: Further studies of effect of endotoxin on antibody response of rabbit to common antigen of *Enterobacteriaceae*. J. Immunol. *98*, 948–957 (1967a)

Whang, H.Y., Neter, E.: Immunosuppression by endotoxin and its lipoid A component. Proc. Soc. Biol. Med. *124*, 919–924 (1967b)

Whang, H.A., Neter, E.: Effect of cholesterol on immunogenicity of common enterobacterial antigen. Proc. Soc. Exp. Biol. Med. *128*, 956–959 (1968a)

Whang, H.Y., Neter, E.: Inhibition by cardiolipin of the antibody response to bacterial antigens. J. Immunol. *100*, 501–506 (1968b)

Whang, H.Y., Neter, E.: Antigen-associated immunosuppressant: effect of serum on immune response. Science *163*, 290–291 (1969)

Whang, H.Y., Neter, E.: Chlorphenesin: an antigen-associated immunosuppressant. Infect. Immun. *2*, 60–64 (1970)

Whang, H.Y., Neter, E.: The common antigen of *Enterobacteriaceae* and its value in taxonomy. Am. Soc. Microbiol. Annu. Meeting Abstr. *M80*, 85 (1972)

Whang, H.Y., Neter, E.: Production of common enterobacterial antigen by members of the family *Enterobacteriaceae*. Experientia *29*, 96–97 (1973)

Whang, H.Y., Lüderitz, O., Westphal, O., Neter, E.: Inhibition by lipid A of formation of antibodies against common antigen of *Enterobacteriaceae*. Proc. Soc. Exp. Biol. Med. *120*, 371–374 (1965a)

Whang, H.Y., Cohen, E., Neter, E.: Immunologic studies of antigenically modified lymphocytes from patients with leukemia. Vox Sang. *10*, 161–168 (1965b)

Whang, H.Y., Yagi, Y., Neter, E.: Characterization of rabbit antibodies against common bacterial antigens and their presence in the fetus. Int. Arch. Allergy Appl. Immunol. *32*, 353–365 (1967)

Whang, H.Y., Chun, D., Neter, E.: Immunosuppression by antigen-associated human serum alpha-one protein. J. Immunol. *103*, 824–827 (1969)

Whang, H.Y., Chun, D., Yagi, Y., Neter, E.: Immunogenicity of soluble and aggregated common enterobacterial antigen. Int. Arch. Allergy Appl. Immunol. *38*, 57–67 (1970)

Whang, H.Y., Mayer, H., Neter, E.: Differential effects on immunogenicity and antigenicity of heat, freezing and alkali treatment of bacterial antigens. J. Immunol. *106*, 1552–1558 (1971a)

Whang, H.Y., Neter, E., Springer, G.F.: Specificity of an erythrocyte membrane receptor for bacterial antigen. Z. Immunitäts.-Forsch. *140*, 298–303 (1971b)

Whang, H.Y., Heller, M.E., Neter, E.: Production by *Aeromonas* of common enterobacterial antigen and its possible taxonomic significance. J. Bacteriol. *110*, 161–164 (1972a)

Whang, H.Y., Mayer, H., Schmidt, G., Neter, E.: Immunogenicity of the common enterobacterial antigen produced by smooth and rough strains. Infect. Immun. *6*, 533–539 (1972b)

Whang, H.Y., Loza, U., Neter, E., Milgrom, F.: Gel precipitation of common enterobacterial antigen by its antibody. Int. Arch. Allergy Appl. Immunol. *45*, 905–914 (1973)

Whang, H.Y., Mayer, H., Schmidt, G., Neter, E.: Strain-related differences in immunosup-

pressive effects of *Enterobacteriaceae* and their lipopolysaccharides on production in rabbits of antibody to enterobacterial common antigen. Infect. Immun. *13*, 1074–1079 (1976)

Wicken, A.J., Knox, K.W.: Lipoteichoic acids: A new class of bacterial antigens. Science *187*, 1161–1167 (1975)

Wilkinson, S.G.: Amino components of the lipopolysaccharide from *Pseudomonas aeruginosa* N.C.T.C. 8505. Presence of 2,4-diamino-2.4.6-trideoxy-D-glucose. Biochem. J. *161*, 103–109 (1977)

Williamson, A.R., Zamenhof, S.: The type-specific substance of *Hemophilus influenzae*, type d: the natural occurrence of glucosamine uronic acid. J. Biol. Chem. *238*, 2255–2258 (1963)

Wu, T.C.M., Park, J.T.: Chemical characterization of a new surface antigenic polysaccharide from a mutant of *Staphylococcus aureus*. J. Bacteriol. *108*, 874–884 (1971)

Young, L.S., Hoffman, K.R., Stevens, P.: "Core" glycolipid of *Enterobacteriaceae:* immunofluorescent detection of antigen and antibody. Proc. Soc. Exp. Biol. Med. *149*, 389–396 (1975)

Zabriskie, J.B.: Mimetric relationships between group A *Streptococci* and mammalian tissues. Adv. Immunol. *7*, 147–188 (1967)

Current Topics in Microbiology and Immunology

Cumulative Author and Subject Index
Volumes 40–85

Springer-Verlag Berlin · Heidelberg · New York 1979

Author Index — Volumes 40–85

Subject Index — Volumes 40–85