Age-Related Cellular Resistance of the Chicken Embryo to Viral Infections
III. *Escherichia coli* resistance-inducing protein[1] (34790)

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Escherichia coli and other Gram-negative bacteria induce antiviral resistance in the chicken embryo and its cells in culture (1). The resistance appears to be mediated partly through the interferon system, since (i) interferon-like activity was recoverable from allantoic and cell culture fluids, (ii) protection was effective against unrelated viruses, (iii) resistance occurred intracellularly during viral replication, and (iv) host synthesis of a protective factor was required for development of resistance.

The exact moiety in the bacterium which elicits the antiviral effect is still unknown. Single-stranded RNA, a normal cellular constituent, can induce interferon formation and antiviral resistance in certain cell cultures (2). However, attempts to isolate from *Brucella* a phenol-extractable nucleic acid which could induce interferon were unsuccessful (3). Endotoxin preparations can also induce interferon, but the active components of these complex cell wall materials has not been definitively established. Feingold *et al.* (4) recently showed that glycolipid from mutants of *Salmonella typhimurium* deficient in cell-wall polysaccharide could still induce in mice the formation of interferon, evidence which they felt implicated lipid as the important moiety. However, the presence of other components in endotoxin preparations cannot be excluded. The observations reported here indicate that *E. coli* protein is essential in induction of interferon-like resistance against viruses in the chicken embryo.

Materials and Methods. Viruses. Newcastle disease virus (NDV), AMS-3 (CG) strain and *neurotropic influenza* virus (A_0/NWS) were prepared as previously described (1).

Bacteria. A heat-killed preparation of *E. coli* 0111:B4:2 was prepared by methods reported previously (1). Two mutants of *E. coli* 0111:B4 were received from E. C. Heath and prepared as described for the wild-type strain. The uridine diphosphate-galactose epimerase-less mutant (J-5) possesses all the phosphorylated heptose region of the lipopolysaccharide core but lacks the core oligosaccharide (5). The RC-59 mutant (genetic defect unknown) lacks the entire core structure and produces a lipopolysaccharide composed only of lipid A substituted with 2-keto-3-deoxyoctonate (KDO) (E. C. Heath, personal communication).

Chicken embryos. The source, incubation, and inoculation of white leghorn fertile eggs were previously outlined (1).

Interference experiments. The protective agents were inoculated intra-allantoically into groups of 15–20 13-day-old embryos. After incubation at 35° for 24 hr, the embryos were challenged intra-allantoically with an appropriate viral dilution to kill 75–100% of the embryos. Embryos were kept thereafter at 35° and candled daily to determine viability. Cumulative percentage mortalities were calculated, and protection was expressed as the percentage difference in mortality between control and experimental groups.

Interfering agents. Boivin or Westphal

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types of *E. coli* 0111:B4 endotoxin from Difco Laboratories were dissolved in 0.15 *N* NaCl to a concentration of 5 mg/ml and heated at 100° for 10 min. Penicillin and streptomycin were added; and aliquots were frozen at —20°. Boivin type endotoxin (6) was also prepared from *E. coli* 0111:B4:2. Endotoxin doses of 500 μg were administered to 13- and 15-day embryos and 25 μg to 7- and 11-day embryos; higher concentrations in younger embryos were toxic.

Control embryos were inoculated with either 0.15 *N* NaCl or GLB medium (containing gelatin and lactalbumin hydrolysate in Hanks' balanced salt solution) (1).

Fractionation of E. coli. *E. coli* 0111:B4:2 was grown with aeration in Difco trypticase soy broth, washed with 0.15 *N* NaCl, heated at 70° for 1 hr, and disrupted by sonication in 50-ml aliquots in a Raytheon sonicator for 30 min. Microscopic examination of sonicated cells revealed no intact bacteria. The sonicated cells were centrifuged sequentially at 1000, 10,000, 40,000, and 100,0000*g* at 4°. For some experiments bacteria were disrupted in a French press and then subjected to differential centrifugation.

Enzymes. The following enzymes were dissolved in 0.15 *N* NaCl: 3× crystallized trypsin, 1 mg/ml (Worthington); *Streptomyces griseus* pronase, B grade, 6.25 mg/ml (Calbiochemicals); crystalline bovine pancreatic deoxyribonuclease I, 500 μg/ml (Worthington); crystalline ribonuclease, 500 μg/ml (Worthington); wheat germ lipase, 500 μg/ml (Mann); partially purified phospholipase C, 500 μg/ml (Worthington); cabbage phospholipase D, 500 μg/ml (Mann); and 3× crystallized alpha chymotrypsin, 5 mg/ml (Worthington). Preparations were sterilized by filtration through Swinnex 0.22-μ filters, and frozen in aliquots at —20°. Twice-crystallized papain, 10 mg/ml (Worthington), was dissolved in 0.15 *N* NaCl with 0.01 *M* EDTA, 0.05 *M* cysteine, penicillin (1000 U/ml), streptomycin (1000 μg/ml), and neomycin (500 μg/ml). Crystalline thermolysin was received from H. Drucker (7).

Materials were treated with enzymes for 3–7 hr at 37° and then dialyzed overnight against 25–50 vol of 0.15 *N* NaCl. Effectiveness of enzymes was monitored by measuring DNA, RNA, and protein before and after treatment.

Protein, RNA, and DNA measurements. Protein concentration was determined by the Folin-Ciocaulteau method of Lowry *et al.* (8) using crystalline bovine plasma albumin (Armour Pharmaceutical) as the standard. RNA concentration was measured by the modified Schmidt-Thannhauser method (9) with yeast ribonucleic acid (Worthington) as the standard. DNA concentration was determined by a fluorometric method (10) with calf thymus DNA (Worthington) as the standard.

Results. Antiviral resistance produced by killed E. coli *and endotoxin.* Antiviral protection elicited by killed bacteria was dose-dependent up to a maximum response (Table I). Boivin or Westphal types of endotoxin prepared from *E. coli* stimulated resistance of 13-day embryos to NDV and NWS viruses, but neither endotoxin preparation was as effective as heat-killed cells (Table I). Endotoxin-induced resistance followed the age-related and virus-related patterns seen for the whole bacterial cells (1). Boivin endotoxin inoculated into 7- and 9-day embryos did not promote resistance against NWS virus, but did stimulate resistance in 13-day embryos. As with heat-killed *E. coli*, endotoxin evoked less, but significant, resistance against vesicular stomatitis virus in embryos of any age.

Activity of cell-wall deficient E. coli *mutants.* Although endotoxin stimulated protection, *E. coli* mutants deficient in cell-wall polysaccharide proved as effective as the wild-type *E. coli* in eliciting resistance to NDV (Table I). Mutant RC-59 which lacked all the lipopolysaccharide core except for KDO and lipid A induced 41% protection while the wild-type strain induced 45% protection.

Fractionation of E. coli. After fractionation by differential centrifugation, considerable protective activity (67%) remained in the final 100,000*g* supernatant fraction of sonicated *E. coli* cells. The remainder of activity was distributed among the other fractions, and remained in sedimentable fractions even

TABLE I. Antiviral Resistance Against Newcastle Disease Virus (NDV) Induced by *E. coli* 0111:B4:2, Endotoxin, and Lipopolysaccharide-Deficient *E. coli* Mutants.

Protective agent	Exp.:	Mortality[a] (%) 1	2	3
Control diluent		95	70	100
Heat-killed *E. coli* 0111:B4:2				
2.5×10^9		21	17	
1.0×10^9				55
8.0×10^8			31	
2.5×10^8			23	
2.5×10^7			54	
E. coli 0111:B4 Boivin endotoxin				
500 μg		57		
50 μg		55		
5 μg		79		
E. coli 0111:B4 Westphal endotoxin				
500 μg		42		
50 μg		52		
5 μg		57		
Heat-killed *E. coli* 0111:B4 Mutant				
J-5[b]				36
RC-59[c]				59

[a] Percentage mortality cumulated after infection with NDV 24 hr after inoculation of protective agents into 13-day embryos.

[b] 1×10^9 cells; contains phosphorylated heptose region of lipopolysaccharide but lacks core oligosaccharide.

[c] 1×10^9 cells; lipopolysaccharide contains only lipid A and 2-keto-3-deoxyoctonate.

after they were washed. The soluble fraction from the *E. coli* RC mutant was also able to protect embryos against NDV and NWS infections. Similar results were obtained with cells disrupted in a French press. The 100,000*g* soluble fraction from *E. coli* 0111:B4:2 was used for further characterization of the protective material.

Protein nature of the active principle. The proteolytic enzyme, pronase, which can hydrolyze most peptide bonds (11) completely abolished the protective ability of the soluble fraction (Tables II and III). Treatment with several other more specific proteolytic enzymes, chymotrypsin, papain, and thermolysin, also reduced antiviral activity of the material (Table II). Ammonium sulfate at 40% saturation precipitated much of the activity of the soluble fraction from which nucleic acids had been removed (Table IV). Antiviral protection thus appeared dependent on protein.

Absence of resistance-inducing nucleic acid. Treatment of the soluble fraction with deoxyribonuclease or ribonuclease removed most of the respective nucleic acid but did not markedly affect the activity of the preparation (Table III). When nucleic acids were removed from the fraction by precipitation with 10% streptomycin sulfate, activity remained in the supernatant (Table IV), indicating that the active principle was not nu-

TABLE II. Reduction of Protective Ability of *E. coli* Soluble Fraction After Treatment with Various Proteolytic Enzymes.

Protective agent	Remaining protein (%)	Mortality (%)[b]
Control		100
Soluble fraction (SF)[a]	100	67
SF + trypsin (100 μg/ml)[c]	85	73
SF + chymotrypsin (250 μg/ml)	43	87
SF + pronase (280 μg/ml)	18	100
SF + papain (500 μg/ml)	39	86
SF + thermolysin (170 μg/ml)	33	80

[a] Soluble fraction obtained from *E. coli* 0111:B4:2 cells disrupted by French press, and sequentially centrifuged to produce a 100,000*g* supernatant. This material contained 140 μg/ml of protein.

[b] Total percentage mortality cumulated after infection with 7 PFU of Newcastle disease virus after inoculation of 13-day embryos with the various materials.

[c] Enzyme treatments were performed for 3 hr at 37°, after which the samples were dialyzed overnight at 4°, against 0.15 N NaCl.

TABLE III. Activity of the Soluble Fraction (SF) from *E. coli* after Various Treatments.

	Remaining (%)			Mortality (%)[a]	
Protective agent	Protein	DNA	RNA	Exp. 1	Exp. 2
Control (C)				79	90
Soluble fraction (SF)[b]	100	100	100	17	58
SF + pronase (625 μg/ml)[c]	20	44	152	91	
C + pronase				100	
SF + ribonuclease (25 μg/ml)	97	57	31	47	
C + ribonuclease				100	
SF + deoxyribonuclease (25 μg/ml)	103	1	23	50	
C + deoxyribonuclease				92	
SF + periodate (0.025 *M*)	76	96	139		44
C + periodate					100
SF + phospholipase C (50 μg/ml)	100	86	60		44
C + phospholipase C					100
SF + phospholipase D (50 μg/ml)	97	91	63		65
C + phospholipase D					95
SF + lipase (50 μg/ml)	99	86	—		67
C + lipase					100

[a] Total percentage mortality cumulated after infection with Newcastle disease virus (NDV) 24 hr after inoculation of 13-day embryos with the various agents.

[b] SF = 100,000*g* supernatant fraction obtained from sonicated heat-killed *E. coli* 0111:B4:2 cells, containing 395 μg of protein, 121 μg of DNA, 120 μg of RNA/ml, small amounts of free fatty acids, sphingosine, and unidentified phospholipids.

[c] Enzyme treatments were all for 3 hr at 37°, after which the materials were dialyzed for 18 hr at 4° against 0.15 *N* NaCl. Periodate treatment was for 18 hr at 4° in the dark, the reaction stopped by addition of 1% glycerol, and the material then dialyzed.

cleic acid. Attempts to induce resistance in the embryo by administration of natural or synthetic ribonucleic acids also failed (12).

Absence of resistance-inducing lipid and carbohydrate. After the soluble fraction was extracted with ethyl alcohol:ethyl ether (3:1), no protection was provided by the ether–alcohol soluble material. However, the nonlipid precipitate from the extraction provided 89% of the activity of the original material. The soluble fraction was also treated with the lipolytic enzymes, lipase, phospholipase C, and phospholipase D, which did not markedly affect activity (Table III). Silica gel thin-layer chromatography detected free fatty acids, sphingosine, and unidentified phospholipids; lipid patterns remained unchanged in the untreated and enzyme-treated materials. Periodate treatment to destroy carbohydrates (containing adjacent–COH groups) in the soluble fraction also did not affect activity (Table III). Thus lipid and carbohydrate did not appear to be major components of the active principle.

Other properties of the resistance-inducing protein. The active moiety of the soluble fraction could be filtered through a 0.22-μ Millipore filter, did not dialyze through Visking cellophane tubing, and was retained by Sephadex G-100. This suggested a probable molecular weight range of 20,000 to 100,000. The protein was heat-stable, either to heating at 60° for 1 hr, or 100° for 10 min.

Discussion. Heat-killed *E. coli*, as well as other Gram-negative bacteria (1), protected chicken embryos against lethal challenge with Newcastle disease and neurotropic influenza viruses. The resistance induced by the bacterium appeared mediated partly by the interferon system (1). The present observations indicate that protection was due to a protein moiety in the bacterium found in the 100,000*g* soluble fraction obtained from disrupted cells.

TABLE IV. Streptomycin Sulfate and Ammonium Sulfate Precipitation of the Antiviral Soluble Fraction from *E. coli.*

Protective agent	Mortality from NDV (%)[a]
Control diluent	95
10% Streptomycin SO_4 (SmS)	85
Soluble fraction (SF)[b]	30
SmS-SF precipitate[b]	76
SmS-SF supernatant[b]	43
SmS-SF supernatant + $(NH_4)_2SO_4$—precipitate[b]	60
SmS-SF supernatant + $(NH_4)_2SO_4$—supernatant[b]	81

[a] Total mortality cumulated after infection with Newcastle disease virus (NDV) 24 hr after inoculation of 13-day embryos with protective agents.

[b] SF = 100,000*g* supernatant obtained from sonicated heat-killed *E. coli* 0111:B4:2 cells. SF-SmS precipitate = precipitate obtained after reaction with streptomycin sulfate at a final concentration of 0.4% for 40 min at 4°, centrifugation at 16,000*g* for 15 min, and dialysis overnight versus 0.15 *N* NaCl. Before inoculation the material was filtered through a 0.22-μ Millipore filter. SF-SmS supernatant = supernatant obtained from above procedure. SF-SmS supernatant + $(NH_4)_2SO_4$—precipitate = precipitate obtained after reaction with $(NH_4)_2SO_4$ at 40% saturation for 40 min at 4°, followed by above centrifugation, dialysis, and filtration. SF-SmS supernatant + $(NH_4)_2SO_4$—supernatant = supernatant obtained after above procedure.

Various protein materials from Gram-negative bacteria have been reported to possess antiviral activity, but their action has not been similar to interferon-mediated resistance. Vilcek and Freer (13) isolated a protein-like fraction from sonicated *E. coli* cells which enhanced the action of interferon. However, the protective effect of *E. coli* in the chicken embryo does not seem to occur by such enhancement (1). A basic phage protein obtained from λ-phage-infected *E. coli* inhibited only DNA viruses, appearing to specifically inhibit viral DNA synthesis; no interferon was induced (14, 15). Another protein obtained from *E. coli* also reduced cytopathic effects of several DNA viruses without affecting RNA viruses, evidently acting by inhibiting viral and cellular DNA metabolism (16). Unlike interferon or the presently described resistance-inducing protein, the agent had to be present during viral replication to be effective.

The present observations appear to be the first indication that a protein can induce interferon-like resistance in the chicken embryo. The component in Gram-negative bacteria or endotoxin responsible for induction or release of interferon in other hosts remains uncertain (17). Westphal glycolipid from a heptoseless *Salmonella* mutant induced interferon as well as did glycolipid from the wild-type strain, a finding which the authors felt indicated the importance of lipid A and KDO (4). Following removal of KDO by acid hydrolysis the remaining material was still able to induce interferon, implicating lipid A as the interferon inducer (18); no measurement of protein was reported. However, as shown by Nowotny *et al.* (19), the Westphal hot phenol method of preparing the endotoxin does not produce a product entirely free of protein. In addition, lipid A is an ill-defined macromolecule in which amino acids, glucosamines, phosphate, acetyl groups, long-chain fatty acids, β-hydroxy-myristic acid, and cations have been found (20). Therefore, protein as well as lipid components might still be possible inducing agents in such preparations.

Two proteins are thought to occur in the cell wall of Gram-negative bacteria, a lipoprotein on the outermost surface and a possibly globular protein attached to the inner peptidoglycan layer which gives the cell wall rigidity (21, 22). The mode of attachment or interlocking of these proteins with other components in the cell wall complex is not clear. Due to repeated shear forces exerted during sonication, fragments of cell-wall material containing protein can become solubilized (23). On the other hand, protein material in the cell cytoplasm may contaminate cell-wall fragments. Rudbach *et al.* (24) were successful recently in obtaining an occult endotoxin in bacterial protoplasm associated with protein; but in contrast to resistance-inducing protein which was destroyed by pronase, they found that the activity of endotoxin was increased following pronase treatment. Our present observations reveal most of the resistance-inducing activity to be in the 100,000*g* soluble fraction, suggesting a protoplasmic source.

Summary. Escherichia coli 0111:B4:2 in doses greater than 10^8 heat-killed cells protected 13-day chicken embryos against challenge with a lethal dose of Newcastle disease virus or neurotropic influenza virus. Intact cells were not required since antiviral resistance was also produced by most *E. coli* fractions, including a 100,000*g* soluble fraction obtained from differential centrifugation of disrupted bacterial cells. The resistance-inducing material appeared to be a protein. Various proteolytic enzymes reduced the antiviral activity of the 100,000*g* soluble fraction, while treatment with lipolytic enzymes or nucleases did not affect activity. The protective ability of this fraction was not extractable into lipid solvents nor was it removed by periodate treatment to destroy carbohydrate. The resistance-inducing moiety was recovered in the precipitates formed by ammonium sulfate or ethanol–ether. *E. coli* mutants deficient in cell-wall lipopolysaccharide provided antiviral resistance equivalent to that produced by the wild-type *E. coli*. It remains uncertain which bacterial structure(s) is the source of the resistance-inducing protein.

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