SKIN-REACTIVE PROPERTIES OF DIFFERENT CELLULAR FRACTIONS OF ANIMAL-PASSED C. Jejuni/Coli

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Abstract

Polymyxin B treated cell sonicates, cell-free supernatants and outer membrane proteins were prepared from animal-passed, virulence-enhanced strains and original isogenic strains of Cjejuni and tested for dermal toxicity in rabbits. Polymyxin B extracts produced the strongest lesions, but the outer membrane protein from 6 times mouse-passed strains elicited a mild skin reaction. Culture-supernatant did not give any noticeable toxicity, suggesting the presence of insufficient factor required to produce measureable effect. Dermal toxicity clearly differentiated animal-passed strains from unpassed original strains which elicited only a weak response(JPMA 37: 132, 1987).

INTRODUCTION

Although campylobacter fejuni/coli is an important cause of acute human enteritis, major questions concerning the possible production of toxins or other factors responsible for the pathophysiology of infections it causes remain unanswered[1]. With the advent of selective plating media and use of microacrophiic conditions for growth, C. jejuni is now being isolated from clinical stool specimens more frequently than Salmonella or Shigella spp.[2,3]

We have recently developed an infant mo use model of gastroenteritis resembling the human disease caused by C.jejuni. This model utilized virulence enhancing iron dextran, mucin and animal-passage to express the virulence of C. /ejuni. During animal-passage, the LD 50 decreased from 10' ° to 105 CFU/ml indicating one million-fold enhancement in virulence. When virulence-enhanced strains were fed to neonatal mice they developed severe diarrhea mimicking human disease.[4] Establishment of a successful enteric infection is dependent on a number of surface components (Virulence factors) of a bacterium for example they mediate attachment to intestinal epithelial cells, prevent removal of organisms by gut motility etc. In order to determine the virulence potential, we compared various cellular fractions isolated from both the animal passed, virulence enhanced strains with corresponding original unpassed strains of C. je/uni/ coli for dermal-toxicity in rabbits by the method of Mageau and Roberson[5].

MATERIALS AND METHODS

a) BACTERIAL CULTURES

clinical isolates, ATCC 29428 and a fresh hospital isolate (G7, Georgetown U. Hosp.) and two animal isolates, one from a chicken, JCH 667 and a second from a pig (C. coli, ATCC 456) were used. These organisms were serially passed in weanling mice with iron dextran or gastric mucin, reducing the dose with each subsequent passage, as previously described. Recovered organisms were retained for comparison with the unpassed strains. Cultures were maintained frozen (-70°C) in Brucella Broth

containing 20% glycerol for use as stocks and subsequent comparisions.

b) PREPARATION OF CELL FREE FILTRATES

Animal-passed strains showing virulence enhancement and original strains were grown in Brucell Broth (Difco) broth with Blaser's selective supplements in flasks incubated at 42°C on a shaker bath. Cells were removed by centrifugation at 6000 x G for 20 minutes at 4°C, the supernatant was sterilized by passage through a 0.22 urn filter, concentrated 10 x by pervaporation, dialyzed against PBS and refiltered.

Cells were iitcubated with Polymyxin B (100 IU./MI) at 37°C for 15 to 20 minutes to release periplasmic proteins, cells were removed by centrifugation (6000 x G for 30 mm); the supernatant was collected. One portion was heated at 56°C for 30 min and the other held at 4°C.

c) PREPARATION OF OUTER MEMBRANE PROTEINS

Outer membrane proteins (OMIP) were prepared using a modification of the method of Blaser et al.[6] Stocks of selected animal-passed, virulence enhanced and original unpassed strains were grown on Brucella Agar with 5% horse blood at 37 C or 42 C and mcubated m an atmosphere with 5% Oxygen, 10% Carbon dioxide and 85% Nitrogen[7] for 20 to 72 h. Cells scrapped from plates were suspended in 20 ml of sterile distilled water and washed once in 0.01 M Tris Buffer pH 7.4 (5000 xg, 10 min at 25"C). The pellet was resuspended in 0.01 M Tris and the density was adjusted to 26% transmission at 450 nm. The suspension was then recentrifuged and the pellet frozen at - 20°C until the membrane preparations were made. Frozen pellets were thawed and resuspended in 20 ml of .01 M Tris Buffer (pH 7.4) and the cells were sonicated on ice for four 30-sec pulses with 30 sec rests. Whole cells were removed by centrifuging the sonicate two times at 6000 xg for 15 min at 4°C. Supemnatant was then centrifuged at 100,000 xg for 1 h at 4°C (in a L870 Ultracentrifuge Backman Instruments, Inc., Fullerton, CA). The pellet was then solubiized by the method of Filip et al.[8] which included incubation of the pellet in 10 ml of 1% sodium lauryl sarcosinate (Sarkosyl; Ciba-Geigy Corp., Greensboro, NC) in 7 mM EDTA for 30 min at 37°C. this suspension was then centrifuged at 100,000 xg for 2 h. The supernatant (now termed as Sarkosyl-soluble) was collected and saved for later studies. The pellet was suspended in 10 mls of Tris buffer and recentrifuged at 100,000 xy for 2 h to remove any Sarkosyl left - The resulting pellet (Sarkosyl - insoluble) was suspended in 1 .0 ml of sterile distified water - Protein concentration of each outer membrane preparation was determined by using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as the standard and stored at 4°C.

d) TOXIC1TY TESTING OF CELLULAR COMPONENTS

The association of toxicity with a particular cellular component was attempted by testing for dermal toxicity of isolated cellular fractions from both the animal-passed, virulence-enhanced and corresponding original strains of C. jejuni as described by the method of Mageau and Rob erson[9]. Intradermal injections of known concentrations of material in a 0.1 ml volume of distilled water were made in the shaved flanks of 6-8 lb albino rabbits. Relative toxicity of sterile whole cell sonicate, cell-free supernatant and purified outer membrane proteins were determined by comparing the character of the lesion produced in rabbit dermis. For comparison a lesion index was calculated by measuring the major and minor axis of the lesion in mm, taking the product of the two and adding one point each for erythema, induration and pus formation. These lesion indexes were determined daily for the first week of the test, then every other day for two weeks. A positive reaction was characterized by a persistent erythematoüs and indurated lesion at least 5 mm In diameter.

RESULTS

DERMAL TOXICITY OF CELLULAR COMPONENTS

Culture supernatants (sterile), from the three strains produced only a primary inflammatory response.

Outer membrane proteins of only the virulence, enhanced G7(7) and 29428(6) strains showed an intermediate virulence response in the form of moderate lesions with erythema, induration and central blanching (Figures 1 and 2).
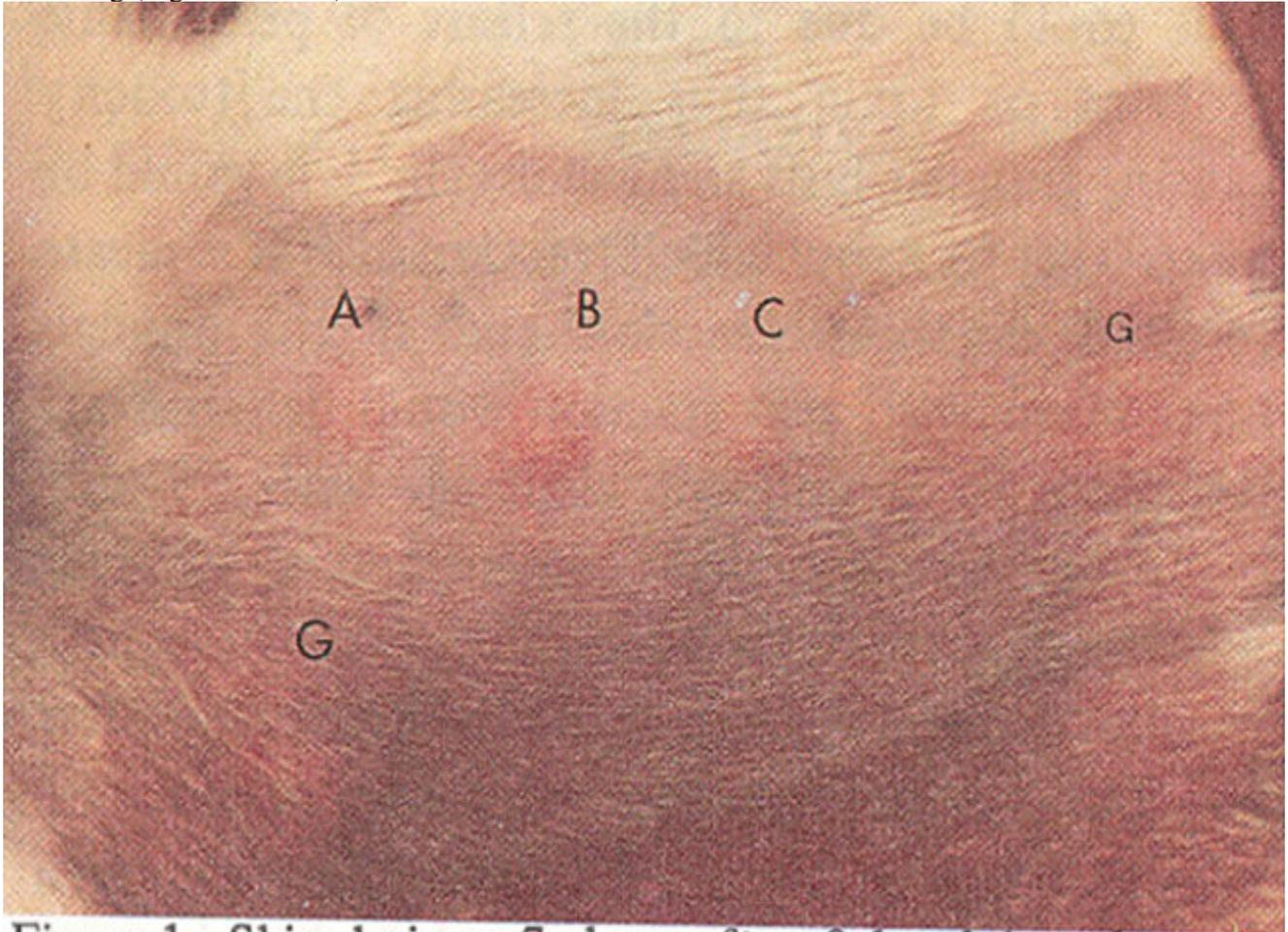

Figure 1. Skin lesions 7 days after 0.1 ml intradermal injection of outer membrane proteins from strains A = G7(0); B = 29428(6); C=ATCC 29428 (0); and G = G7(7).
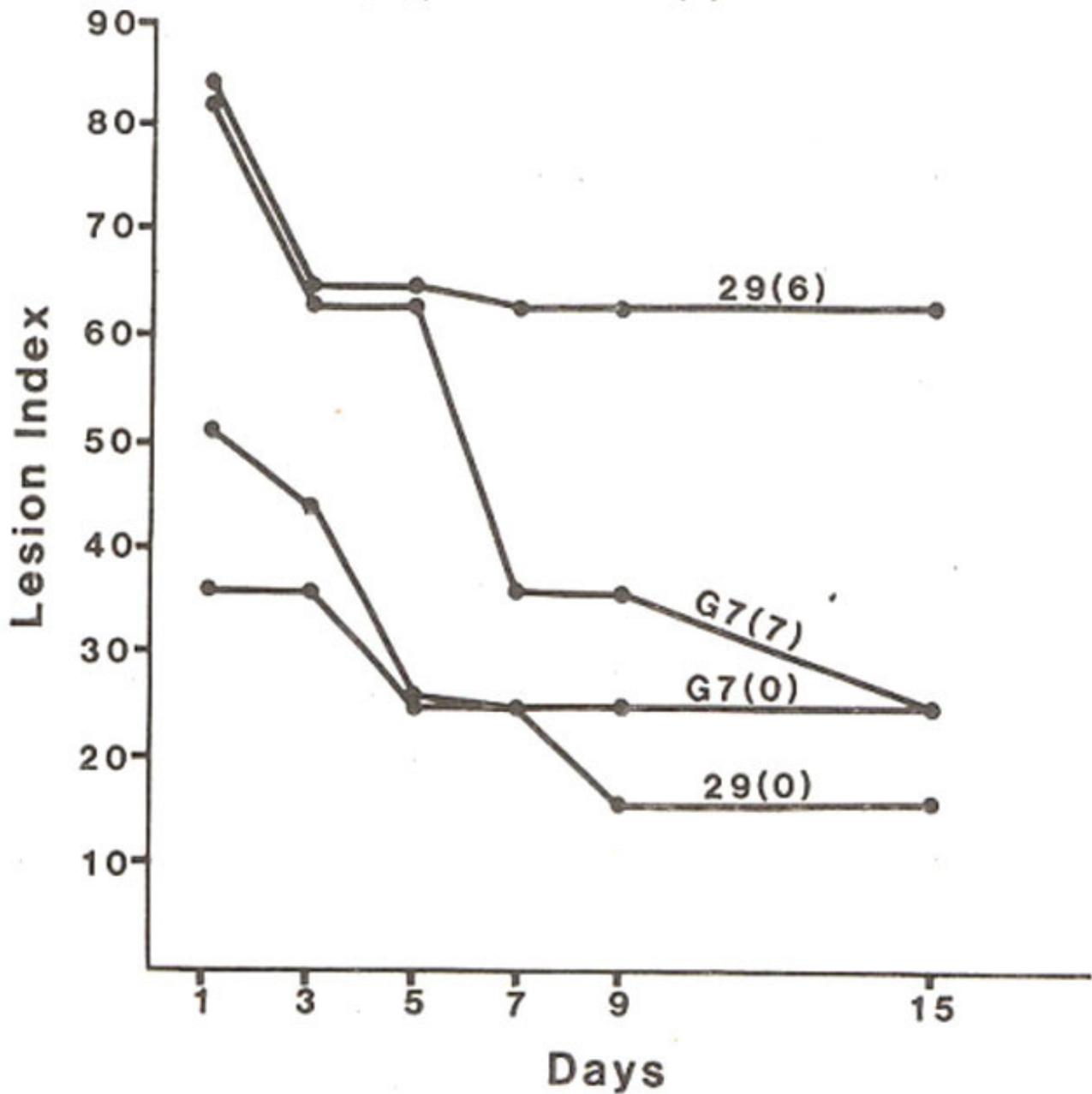
Figure 2. The response of rabbits to a 0.1 ml intradermal infection of outer membrane protein of *C. jejuni* strains: G7(0); G(7)7; ATCC 29428 (0) ATCC 29428 (6).

The lesions regressed during the next 25 days to discrete nodules with central scarring. Sterile sonicates of (Polymyxin B treated cells) strains 456(5), 29428(5), G7(7) gave the strongest reactions The response of these strains was in the form of erythema with increasing induration, which gradually regressed during the next 4 to 6 weeks. Strain G7(0), 456(0) and 29428(0) showed a mild reaction by the production of smaller lesions which subsided to minimum nodules in less than a week (Figures 3 and 4).
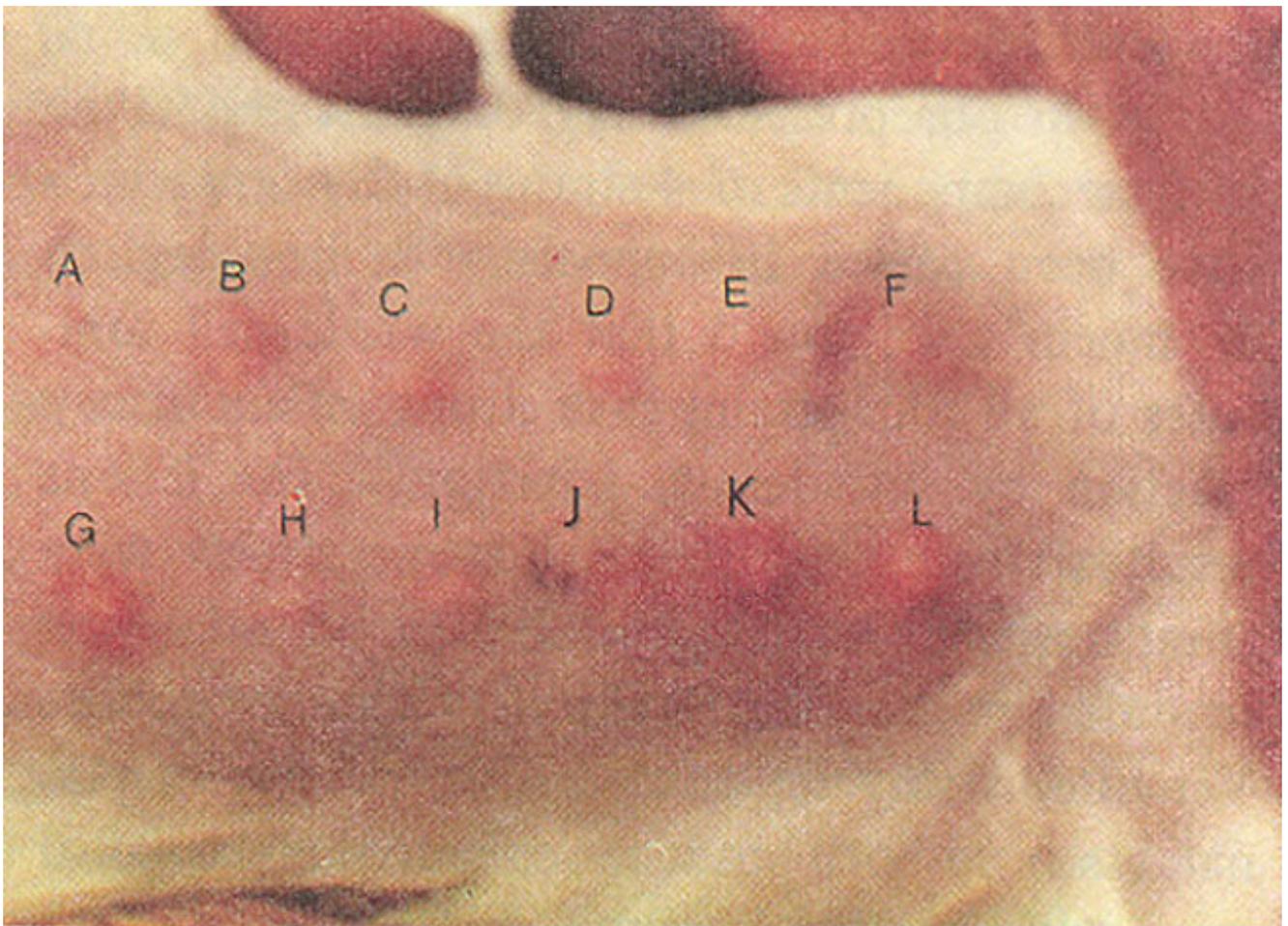
Figure 3. Skin lesions 7 days after 0.1 ml intradermal injection of sterile cell-free sonicates of *C.jejuni* strains: A H$_2$O; B=G7(7); C=G7(0); E=JCH667; F = JCH (6); G = 29428 (6); H = 29428 (0); I = 456 (0); J, K, L = 456 (6).
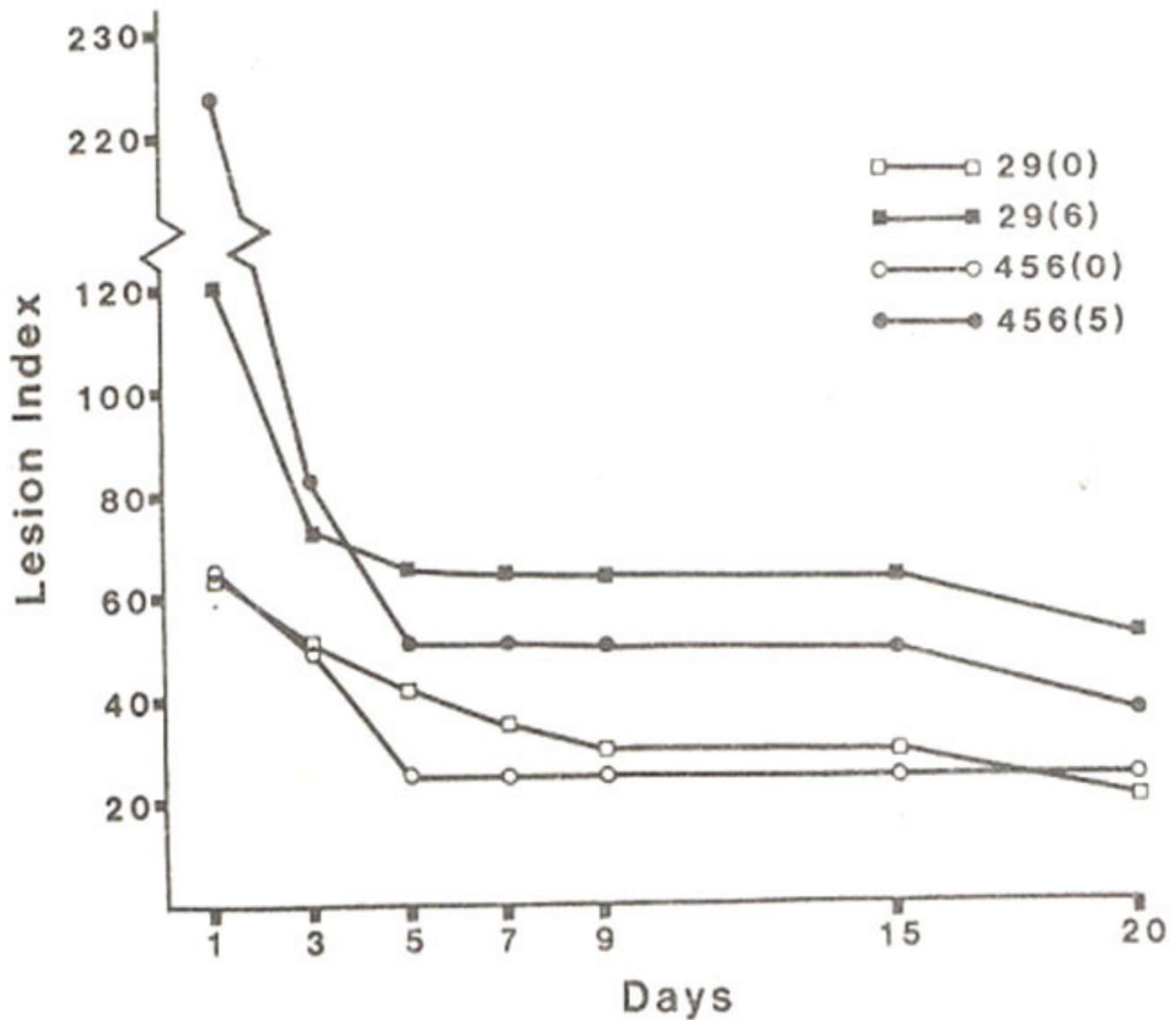
Figure 4. The response of rabbits to a 0.1 ml intradermal infection of cell-sonicates of strains. 29428 (6); 29428(0); 456 (0) and 456 (5).

Since an equal amount of each strain was injected, it is reasonable to assume that different levels of toxicity were' definite expressions of the level of virulence of the animal-passed strains compared with the unpassed strains. The rabbit (Figures 1 & 3) shows typical response of normal animals 72 has after intradermal injection of fractions of C, jejuni. Time of onset of the lesions after injection of the sterile cell sonicates, clearly differentiated the animal-passed from unpassed C. fe/uni strains. In animals injected with original unpassed strains, there was little or no visible sldn reaction except for slight erythema during first 48 hours. However, the response with mouse-passed strains was most intense at 72 has which gradually diminished in most rabbits during the next 4 to 6 weeks. This would suggest an enhanced expression of certain surface components (virulence factors) of Campylobacters during passage with iron-dextran.

DISCUSSION

C. jejuni is now recognised worldwide as a major cause of human enteritis. The exact mechanism (s) by which C. /ejuni causes disease has been and still under investigation. Studies of the structure of campylobacter cell at the molecular level have for the most part focussed on the structure of the cell-surface. This is because, for pathogenic bacteria, it is this surface that interacts directly with the human host and allows the successful pathogen to avoid or to overcome the host defence mechanisms.[10]

In our present study to characterize the surface components of 4 strains of C. fe/uni which have been passed in animals with iron-dextran or. mucin[4], we found that the whole cell sonicates of all four strains gave strong inflammatory reaction by intradermal injection. The lesions resulting from the original isogenic strains were comparatively small and subsided to minimum modules in less than a week. In contrast to the Vero Cell Assay reported earlier[11] culture supernatants from both virulence-enhanced and original strains when tested in rabbit dermis did not give a significant reaction. Dermal toxicity clearly differentiated the virulent from the original unpassed strains.

As yet the nature of skin-reaction in normal rabbit has not been explained. Trust et al.[12] have demonstrated endotoxicity of spontaneously released outer membrane proteins, which are rich in Lipopolysacchride (LPS), from C. jefuni strain VC 74 by mouse lethality assay[12]. The role of LPS in Campylobacter pathogenesis awaits clarification, but the lipid A portion of the molecule appears similar to that of other gram negative bacteria.[13] The endotoxic component in addition to some other factors present in C. fejuni sonicates may be responsible for intense inflammatory action. If this is true then both the original and animal-passed strains should elicit similar skin-reaction. It is possible that virulent strains possess unique O-antigemc determinantsor outer membrane proteins[11] whose expression is enhanced in the presence of iron-dextran during animal passage[4]. Strong skin reacting of Polymyxn B treated cell-sonicates correlated well with our previous observation with Vero Cells. This could be due to the release of periplasmic proteins or toxin thus increasing the toxicity causing granular lysis and cell death.[11] It would be interesting to know which fraction of this sonicate resulled in this reaction and if the animals immunized with that fraction would demonstrate resistance to subsequent challenge with C.jefuni.

In summary we can suggest a working hypothesis. 'that the lesion formation which usually does, not occur until the third day after infection, is actually an immune, phenomenon involving locally deposited Campylobacter antigen (s) and an immune mechanism of the host. Histopathologic and other studies of the skin reactions of normal rabbits to cellular fractions from mouse-passed and unpassed original strains of C. fejuni will be necessary to elucidate the quantitative and qualitative nature of this phenomenon. This information would be of great help in developing procedures for immunoprophylaxis against Campylobacter infections.

REFERENCES

1. Manninen, K.l., Prescott, JF. and Dohoo, 1.R. Pathogenicity of Campylobacter fejuni isolates from animals and humans. Infect. Immun, 1982; 38:46.
2. Blaser, M.J., Berkowitz, I.D., LaForce, F.M., Cravens, J., Reller, L.B. and Wang, W.L. Cam pylobacter enteritis; clinical and epidemiologic features. Ann. Intern. Med., 1979; 91: 179.
3. Blaser, MJ., Reller, L.B., Luechtefeld, N.W. and Wang, W.L. Campylobacter enteritis in Denver. West. J.Med., 1982;136: 287.
4. Kazmi, S.U., Roberson, B.S. and Stern, NJ. Animal-passed, virulence-enhanced Campylobacter jejuni muses enteritis in neonatal mice. Curr. Microbal., 1984; 11: 159.
5. Gianella, R.A, Pathogenesis of acute bacterial diarrhea! disorders. Annu. Rev. Med., 1981; 32: 341.

6. Mageau, R.P. and Roberson, BS. Association of toxic capsule and cell wall mucopeptide with virulence in Gaffkya tetragena. J. Bacteriol., 1969; 97:16.

7. Refe, A.D. and Keush, G.T. Shigella, dysenteriae Cytotoxin; periplasmic protein releasable by Polymyxin B and osmotic shock. Infect. Immun., 1983;43: 270.

8. Blaser, M.J., Hopkins, J.A., Berka, R.M., Vasil, M.L. and Wang, W.L. Identification and characterization of Campylobacter /ejuni outer membrane proteins. Infect. lmmun., 1983; 42:276.

9. Filip, C., Fletcher, G., Wuiff, J.L. and Earhart, C. F. Solubilization of the cytoplasmic membrane of Escherichia coil by the ionic detergent sodiumlauryl sarcosinate. J. Bacteriol., 1973; 115: 717.

10. Smith, H. Microbial surfaces in relation to path6-genicity. Bacteriol. Rev., 1977; 41:475.

11. Kazmi, S.U., Roberson, B.S. and Stern, NJ. Virulence factors expressed by Campylobacter jeuni/coli after passage in BALB/C mice. Abstr. Am. Soc. Microbiol., 85th Annual Meeting, Paper B 206, 1985.

12. Trust, T. J. and Logan, S.M. Outer membrane and surface structure of Campylobacter jejuni, p. 133-142. In J. -P. Butzler (ed.), Campylobacter infection in man and animals. CRC Press, Inc. Boca Raton, FL, 1984.

13. Blaser, MJ., Hopkins, J.A. and Vasil, M.L. Campylobacter fejuni outer membrane proteins are antigenic for humans. Infect. Immun., 1984;43: 986.