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# Immunological Properties of *Actinomyces viscosus*: Comparison of Blastogenic and Adjuvant Activities

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A water-soluble extract of *Actinomyces viscosus* was tested for its capacity to induce deoxyribonucleic acid synthesis in spleen cells from normal mice and to augment the in vitro antibody-forming cell (AFC) response to the T-cell-dependent antigen, sheep erythrocytes (SRBC) by T-cell-deficient adherent spleen cell monolayers from the same mice. Our results indicated that the water-soluble extract induced an in vitro blastogenic response, as assessed by tritiated thymidine incorporation into mouse spleen cells. The water-soluble extract also augmented the antibody response to SRBC. However, after fractionation of the water-soluble extract on Biogel P-300, not all of the fractions induced a blastogenic response and augmented an AFC response to SRBC. This difference in the ability of *A. viscosus* fractions to induce in vitro responses suggests that the water-soluble extract has multiple immunological properties. Chemical analysis of the water-soluble extract indicated an enrichment for amino acids and amino sugars common to peptidoglycan.

Chronic periodontal disease is usually associated with the accumulation of a microbial dental plaque (DP). Exposure of the gingiva and the periodontium to DP results in inflammation, formation of periodontal pockets, and resorption of alveolar bone.

The bacterial component of the subgingival microflora appears to be a major etiological agent in periodontal disease (14, 32). One of these bacteria, *Actinomyces viscosus*, has been shown to be a major component of subgingival plaque (38). Moreover, the presence of *A. viscosus* in a hamster model is associated with periodontal disease (17). We have previously shown that DP has adjuvant activity (9). It is therefore likely that *A. viscosus*, as a major component of subgingival plaque, contributes to gingival inflammation by serving as a direct irritant of the adjacent tissue or as a nonspecific adjuvant to the local lymphoid elements or both. Both nonspecific activation (5, 13) and specific activation of lymphocytes (4, 27) by *A. viscosus* have been reported. Therefore, we have examined the ability of a water-soluble extract of *A. viscosus* (AVS) to induce lymphocyte activation and have found that the extract is capable of inducing an in vitro blastogenic response and augmenting the antibody response to an unrelated thymic-dependent antigen, sheep erythrocytes (SRBC). We have also analyzed the AVS for its chemical composition and found that components common to peptidoglycan are present. The possible relationship between our results and peptidoglycan is discussed.

## MATERIALS AND METHODS

**Animals.** Female C57BL/6 mice were obtained from ARS Sprague Dawley (Madison, Wis.). The mice were housed in plastic cages, bedded on hardwood chips, and covered with filter bonnets. The caged mice were fed Charles River autoclavable mouse chow and water ad lib and were used at 8 weeks of age or older.

**Protein determination.** Total protein nitrogen of DP, an AVS, and AVS fractions were determined by the method of Lowry et al. (21) by using bovine serum albumin as the standard.

**Antigens, adjuvants, and mitogens. (i) SRBC.** SRBC were obtained in Alsevers solution from the Colorado Serum Co. (Denver, Colo.). The SRBC were prepared for use in an in vitro tissue culture system by washing them three times with minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) and suspending them to 20% (vol/vol) in the culture medium described by Mishell and Dutton (24).

**(ii) DP.** DP was collected and prepared as previously described (9). Briefly, DP was collected, pooled, washed in phosphate-buffered saline (PBS; 0.05 M; pH 7.2) and sonicated for 10 min with a Branson sonifier at 4°C. The sonicate was centrifuged at 10,000 × *g*, and the soluble supernatant was sterilized by filtration through a 0.45-μm filter (Nalge/Sybron Corp., Rochester, N.Y.). This sterile supernatant (DP) was adjusted to 150 μg of protein/ml of PBS and stored at −20°C until required.

**(iii) *A. viscosus*.** *A. viscosus* (ATCC 19246) was grown in 9-liter volumes of diffusate medium (22) at 37°C for 72 h. The *A. viscosus* isolates were harvested

by centrifugation, washed three times with sterile PBS, and stored at −20°C. When required, the *A. viscosus* isolates were thawed, resuspended at 100 mg (wet weight)/ml of PBS, and sonicated in a rosette sonifier cell immersed in an ice bath with a Heat Systems Sonifier (Branson model 350) until 95% breakage of the *A. viscosus* cells was obtained, as assessed by phase-contrast microscopy. The water-soluble sonicate was separated from the *A. viscosus* pellet by centrifugation at 10,000 × *g* for 30 min at 4°C. The resulting *A. viscosus* sonicate supernatant (AVS) was sterilized by filtration through a 0.45-μm filter (Nalge/Sybron Corp.) and stored at −20°C until required. The AVS was tested for adjuvant activity at a final concentration of 12.5 μg of protein per spleen cell monolayer. The AVS was also tested for its ability to stimulate deoxyribonucleic acid (DNA) synthesis at concentrations from 0.2 to 20 μg of protein per spleen cell culture.

**Chemical analysis of AVS.** Amino acids were determined with an amino acid analyzer (Beckman amino acid analyzer, model 120C, Beckman Instruments, Palo Alto, Calif.). A 1-mg amount of AVS per ampoule was hydrolyzed in evacuated ampoules with 6 N HCl for 28 h at 100°C. The HCl was removed by evaporation. Hexosamines were determined by the amino acid analyzer after hydrolysis in 1 N HCl at 100°C for 6 h and elution from Dowex X-4. Neutral sugars were determined with a Technicon carbohydrate analyzer (Technicon, Tarrytown, N.Y.) after hydrolysis in 1 N HCl for 6 h at 100°C and subsequent passage through coupled columns of Dowex X-4 and Dowex 1-X8.

**Fractionation of AVS.** A 90-ml sample of AVS was subjected to gel filtration chromatography on a Biogel P-300 column (5 by 100 cm; Biorad, Rockville Center, N.Y.). Fractions (15 ml) were eluted in PBS (pH 7.5, 0.15 M NaCl-0.01 M $PO_4^{\equiv}$) by ascending flow. Protein and carbohydrate concentrations (12) of each fraction were determined. The fractions were divided into six separate pools, dialyzed against distilled water, and then lyophilized. Each pool was reconstituted in 20 to 25 ml of PBS and stored at −20°C until required. Five of six pools were tested for their ability to act as an adjuvant or to stimulate DNA synthesis or both.

**PHA.** Phytohemagglutinin (PHA) was obtained from Burroughs Wellcome Co., (Research Triangle Park, N.C.). The lyophilized lectin was reconstituted at 1 mg/ml of sterile PBS and stored at −20°C. When required, PHA was diluted to 50 μg/ml of RPMI 1640 medium and added in 20-μl volumes to the appropriate cell cultures.

***Escherichia coli* O111:B4 endotoxin (LPS).** Lipopolysaccharide (LPS) was obtained from Difco (Detroit, Mich.). The lyophilized LPS was reconstituted at 1 mg/ml of PBS and diluted to 100 μg/ml of RPMI 1640 when required and added in 20-μl volumes to the appropriate cell cultures.

**Cell cultures. (i) Spleen cell suspensions.** Spleens were obtained from C57BL/6 mice. Spleen cell suspensions were prepared by forcing the organs through a sterile, stainless-steel mesh screen. Cell clumps and debris were removed by gravity sedimentation for 10 min. The cells that were obtained were washed three times with minimal essential medium supplemented with gentamicin (Schering Corp., 50 μg/ml of minimal essential medium). The cell concentration was determined with a Coulter Counter (Coulter Electronics, Hialeah, Fla.). The percent viability of the cell suspension was determined by trypan blue dye exclusion.

**(ii) Preparation of T-cell-deficient mouse spleen cell monolayers.** Spleen cells were resuspended at $10 \times 10^6$ cells per ml of Mishell and Dutton medium (24). Adherent spleen cell monolayers were prepared by the method of Mosier (25). The cells that constitute the adherent cell monolayer are 60% immunoglobulin positive and 45% esterase positive and do not respond to PHA, as assessed by tritiated thymidine ([$^3$H]TdR) incorporation. Furthermore, it is known that adherent cell monolayers prepared by this procedure contain macrophages (25) and antibody-forming cell precursors (20). The inability of this adherent cell population to respond to the T-cell-dependent antigen, SRBC, is therefore due to the absence of significant numbers of T-cells.

**(iii) In vitro induction of the antibody response to SRBC.** The adherent spleen cell monolayers, incubated with or without DP, AVS, or AVS fractions 1, 2, 3, 4, and 5 were immunized with $5 \times 10^6$ SRBC and incubated for 4 days at 37°C in a 10% $CO_2$-7% $O_2$-83% $N_2$ gas mixture on a rocking platform, as described by Mishell and Dutton (24). A parallel set of adherent cell monolayers, incubated with or without DP, AVS, or AVS fractions 1, 2, 3, 4, and 5, but without SRBC, was also prepared and incubated for 4 days under identical culture conditions.

**Enumeration of AFC.** After day 4 of incubation, the adherent cell monolayers were harvested and tested for the presence of cells secreting immunoglobulin M antibody to SRBC by a modification (28) of the Jerne hemolytic plaque-forming cell assay (16). The results are expressed as mean antibody-forming cells (AFC) per culture ± the standard error (SE).

**In vitro blastogenesis.** The ability of splenic lymphocytes to undergo DNA synthesis in response to DP, AVS, and AVS fractions 1, 2, 3, 4, and 5 was assessed by measuring the uptake of [$^3$H]Tdr. Spleen lymphocytes were cultured by the method of Strong et al. (35) for 3 or 6 days. Six hours before harvest on day 3 or 6, 0.2 μCi of [$^3$H]Tdr (specific activity, 2.0 Ci/mM; Amersham Searle, Arlington Heights, Ill.) was added to each well. The cells were harvested on glass fiber filters with a Skatron cell harvester (Flow Labs, Rockville, Md.), and the degree of incorporation of [$^3$H]Tdr was assessed for each culture by liquid scintillation spectrometry with a Beckman LS 3133T scintillation counter. Results are expressed as mean counts per minute ± SE for triplicate cultures or as the stimulation index of mean counts per minute of cells cultured with a stimulant to mean counts per minute of cells cultured without a stimulant.

## RESULTS

**Biochemical properties of AVS.** The AVS was analyzed for amino acid, amino sugar, and neutral sugar content. The results are shown in

Table 1. The AVS was found to have rhamnose, 6-deoxytalose, ribose, mannose, and galactose. The AVS was enriched for glucosamine, glutamic acid, alanine, lysine, and ornithine. These components are commonly found in peptidoglycan. We then assayed for muramic acid concentration (19) and glucosamine concentration (35). The glucosamine concentration was similar to that obtained from the amino acid analyzer (i.e., 56 μg/ml). Muramic acid was also present to a maximal concentration of 23 μg/ml.

**Immunological activities of AVS.** To determine the immunological activities of AVS, two types of experiments were performed. The first series of experiments was designed to test the capacity of AVS to induce blastogenesis in mouse spleen cells. As positive controls, we also simultaneously determined blastogenic activity of PHA, LPS, and DP. We have previously shown that DP can induce a mitogenic response in mouse thymocytes (10). The blastogenic responses of mouse lymphocytes to PHA, LPS, DP, and AVS are presented in Table 2. The results show that AVS stimulated a 3- and a 6-day blastogenic response. Similar, although quantitatively greater, responses were obtained with DP. The blastogenic responses of PHA and LPS were positive at day 3 of incubation and negative at day 6 of incubation (Table 2).

TABLE 1. *Chemical analysis of A. viscosus sonicate*

| Analysis of: | Amt of component (μg/ml) |
|---|---|
| Carbohydrate[a] (total 295.6 μg/ml) | |
| Rhamnose | 42.2 |
| 6-Deoxytalose | 81.6 |
| Ribose | 50.3 |
| Mannose | 17.6 |
| Fucose | 0 |
| Galactose | 20.2 |
| Xylose | 5.0 |
| Glucose | 6.1 |
| Glucosamine | 55.3 |
| Galactosamine | 17.3 |
| Protein[b] (total 80.6 μg/ml) | |
| Aspartic acid | 3.5 |
| Threonine | 1.7 |
| Serine | 0 |
| Glutamic acid | 9.0 |
| Proline | 4.5 |
| Glycine | 1.7 |
| Alanine | 7.3 |
| Half-cystine | 0 |
| Valine | 2.0 |
| Methionine | 0 |
| Isoleucine | 1.1 |
| Leucine | 2.3 |
| Tyrosine | 0 |
| Phenylalanine | 1.0 |
| Lysine and ornithine | 6.4 |
| Histidine | 0.9 |
| Arginine | 3.1 |

[a] Hexosamines from amino acid analyzer after hydrolysis in 1 N HCl at 100°C for 6 h and elution from Dowex 50-X4. Neutral sugars from the carbohydrate analyzer after hydrolysis in 1 N HCl at 100°C for 6 h and subsequent passage through coupled columns of Dowex 50-X4 and Dowex 1-X8.

[b] From amino acid analyzer after hydrolysis in 6.0 N HCl at 105°C for 28 h, evacuated with nitrogen.

The second series of experiments examined the ability of AVS to augment the antibody response of T-cell-deficient mouse spleen cells to the antigen SRBC. Our results indicate that AVS augments the production of antibody to SRBC by adherent spleen cells (Fig. 1). The polyclonal activity of this water-soluble preparation of AVS is minimal in the induction of an AFC response to SRBC. We have also compared the AVS to DP for its ability to augment the AFC response of this T-cell-deficient population of spleen cells to SRBC (Fig. 2) and have found the AVS to be more potent than DP, unlike the blastogenic results that were obtained.

**Gel filtration of AVS.** The AVS was subjected to gel filtration chromatography on Biogel P-300. Fractions were collected in 15-ml volumes. Protein and carbohydrate concentrations were determined for each fraction (Fig. 3), and the fractions were divided into six pools. Five of six pools were tested for their ability to induce DNA synthesis in a population of spleen cells or to augment an AFC response to SRBC in a population of T-cell-deficient spleen cells or both. The AVS fractions 3, 4, and 5 were able to induce a 3-day and a 6-day blastogenic response, as assessed by [$^3$H]Tdr incorporation (Tables 3 and 4). In the initial experiment, fractions 1 and 2 induced a 3-day response but did not stimulate DNA synthesis at day 6 of incubation (Table 3).

TABLE 2. *Blastogenic responses of mouse spleen lymphocytes to DP and AVS*[a]

| Stimulant | 3-Day response | | 6-Day response | |
|---|---|---|---|---|
| | Mean cpm ± SE[b] | SI[c] | Mean cpm ± SE[b] | SI[c] |
| Medium | 851 ± 130 | | 1,179 ± 320 | |
| PHA | 18,290 ± 1,805 | 21.5 | 190 ± 20 | 0.2 |
| LPS | 18,743 ± 751 | 22.0 | 1,366 ± 344 | 1.2 |
| DP | 27,677 ± 2,172 | 32.5 | 14,704 ± 792 | 12.5 |
| AVS | 11,961 ± 1,015 | 14.0 | 7,270 ± 899 | 6.2 |

[a] Mouse spleen cell cultures (5 × 10$^5$ cells/culture) were incubated for 3 or 6 days with medium, PHA (1.0 μg/culture), LPS (2.0 μg/culture), DP (3.0 μg/culture), or AVS (2.0 μg/culture).

[b] Mean counts per minute ± standard error of triplicate cultures.

[c] Stimulation index or ratio of [$^3$H]Tdr incorporated into cells cultured with a stimulant to cells with medium alone.

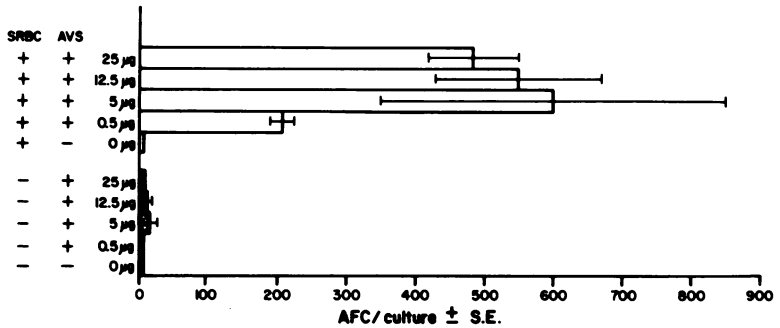


FIG. 1. *Effect of various amounts of AVS on the in vitro AFC response of adherent T-cell-deficient mouse spleen cells to SRBC. Adherent spleen cell monolayers were prepared and incubated with the concentrations of AVS per culture as noted, with or without SRBC. All of the cultures were assayed for anti-SRBC immunoglobulin M-secreting AFC after 4 days of incubation.*

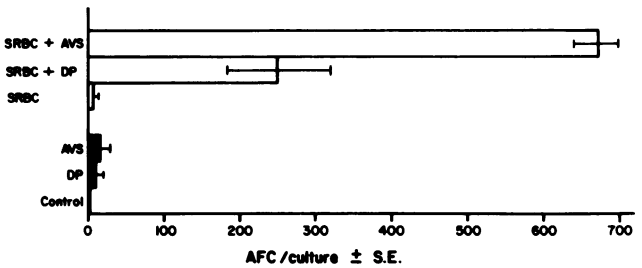


FIG. 2. *Restoration of the in vitro AFC response of adherent T-cell-deficient mouse spleen cells to SRBC by DP and AVS. Adherent spleen cell monolayers were prepared and incubated with various combinations of DP (7.5 µg/culture), AVS (12.5 µg/culture), and SRBC. All of the cultures were assayed for anti-SRBC immunoglobulin M-secreting AFC after 4 days of incubation.*

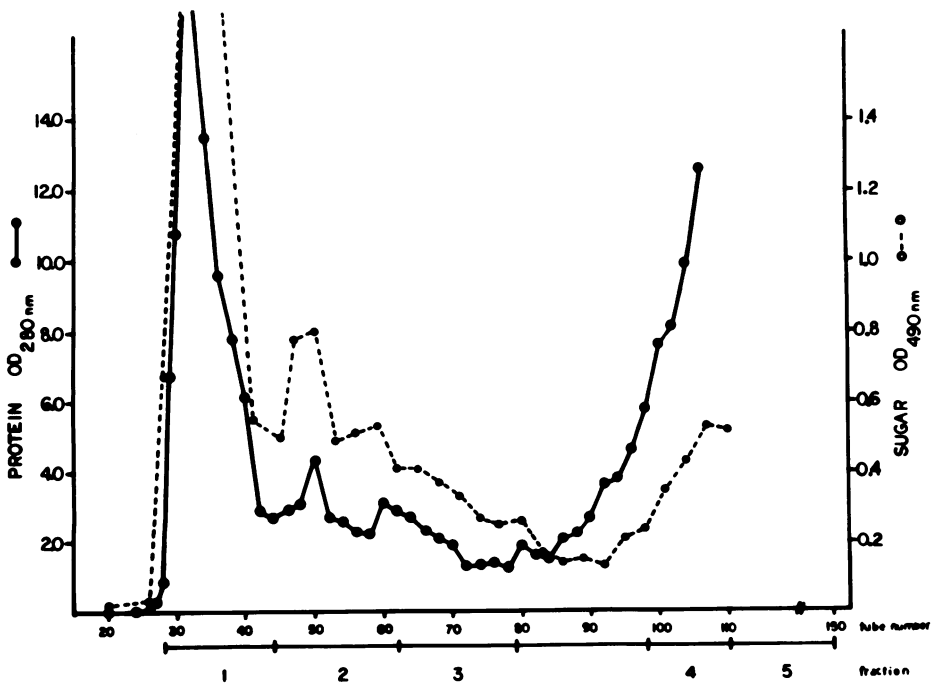


FIG. 3. *Chromatographic profile of AVS on Biogel P-300. Fractions (15 ml) were assayed for protein (●) and carbohydrate (○) and combined into six pools. Five pools were assayed for their ability to induce a blastogenic response or restore the AFC response of a population of T-cell-deficient spleen cell monolayers to SRBC or both. OD, Optical density.*

However, in subsequent experiments that were conducted with material that had been stored at −20°C, fractions 1 and 2 induced both a 3-day and a 6-day blastogenic response. The results of five experiments, conducted on material stored at −20°C, are presented as the mean counts per minute ± the standard error of the mean (Table 4).

We then investigated whether fractions of AVS were able to augment the AFC response to SRBC of adherent spleen cells when compared with DP and AVS. Our results (Fig. 4) indicate that the ability of AVS to augment an AFC response to SRBC is not present in all fractions. This observation was not affected by prior storage of the fractions at −20°C. Fractions 1, 2, 3, and 5 were able to replace the ability of the T-cell-deficient adherent spleen cells to produce antibody to SRBC, but not to the degree of the intact AVS. In contrast, fraction 4 did not augment the AFC response to SRBC.

TABLE 3. *Blastogenic responses of mouse spleen cells to AVS and AVS fractions before storage of the AVS preparations at −20°C*[a]

| Stimulant | 3-Day response | | 6-Day response | |
|---|---|---|---|---|
| | Mean cpm ± SE[b] | SI[c] | Mean cpm ± SE[b] | SI[c] |
| Medium | 833 ± 152 | | 570 ± 81 | |
| PHA | 18,743 ± 628 | 22.5 | 26 ± 6 | 0.04 |
| LPS | 28,954 ± 3,295 | 34.8 | 377 ± 138 | 0.7 |
| DP | 9,404 ± 1,124 | 11.3 | 2,030 ± 850 | 3.6 |
| AVS | 6,285 ± 518 | 7.5 | 5,532 ± 526 | 9.7 |
| AVS fraction 1 | 11,194 ± 409 | 13.4 | 836 ± 253 | 1.5 |
| AVS fraction 2 | 9,720 ± 871 | 11.7 | 157 ± 53 | 0.3 |
| AVS fraction 3 | 6,223 ± 472 | 7.5 | 1,745 ± 91 | 3.1 |
| AVS fraction 4 | 6,046 ± 269 | 7.2 | 4,376 ± 481 | 7.7 |
| AVS fraction 5 | 5,711 ± 311 | 6.8 | 2,737 ± 740 | 4.8 |

[a] Mouse spleen cell cultures (5 × $10^5$ cells/culture) were incubated with medium, PHA (1.0 μg/culture), LPS (2.0 μg/culture), DP (3.0 μg/culture), AVS (2.0 μg/culture), or AVS fractions (2.0 μg/culture) for 3 or 6 days.

[b] Mean counts per minute ± standard error of triplicate cultures.

[c] Stimulation index or ratio of [$^3$H]Tdr incorporated into cells cultured with a stimulant to cells cultured with medium alone.

## DISCUSSION

Dental plaque and its soluble products may contribute to the inflammatory lesion associated with periodontal disease. *A. viscosus* is a major bacterial component of gingival plaque. We have, therefore, tested the ability of a water-soluble extract of *A. viscosus* (AVS) to stimulate DNA synthesis or to augment an AFC response to SRBC or both.

The results of this study have clearly shown that AVS can induce DNA synthesis in mouse splenic lymphocytes. Our results, therefore, support the report of Engel et al. (13) on the mitogenic properties of *A. viscosus* for spleen cells from germ-free mice and the report of Burckhardt et al. (6) on the blastogenic responses of nude mouse spleen cells to *A. viscosus*. However, the results that were obtained in this study at day 6 of incubation would indicate that there

TABLE 4. *Blastogenic responses of mouse spleen lymphocytes to AVS and AVS fractions after storage of the fractions at −20°C*[a]

| Stimulant | 3-Day response | | 6-Day response | |
|---|---|---|---|---|
| | Mean ± SEM[b] | SI[c] | Mean ± SEM[b] | SI[c] |
| Medium | 408 ± 152 | | 197 ± 125 | |
| PHA | 9,570 ± 548 | 23.4 | 42 ± 10 | 0.2 |
| LPS | 8,217 ± 2,608 | 20.1 | 228 ± 116 | 1.2 |
| DP | 6,139 ± 2,585 | 15.0 | 3,338 ± 1,293 | 16.9 |
| AVS | 4,056 ± 1,538 | 9.9 | 1,398 ± 342 | 7.1 |
| AVS fraction 1 | 5,621 ± 2,620 | 13.8 | 2,247 ± 1,384 | 11.4 |
| AVS fraction 2 | 2,809 ± 1,529 | 6.9 | 1,518 ± 672 | 7.7 |
| AVS fraction 3 | 3,584 ± 1,632 | 8.8 | 1,149 ± 463 | 5.8 |
| AVS fraction 4 | 2,190 ± 1,105 | 5.4 | 1,484 ± 1,002 | 7.5 |
| AVS fraction 5 | 3,013 ± 1,228 | 7.4 | 1,489 ± 706 | 7.6 |

[a] Blastogenic responses of mouse spleen lymphocytes to PHA (1.0 μg/culture), LPS (2.0 μg/culture), DP (3.0 μg/culture), AVS (2.0 μg/culture), or AVS fractions (2.0 μg/culture).

[b] Mean ± standard error of mean for five experiments.

[c] Mean of stimulation indexes for five experiments.

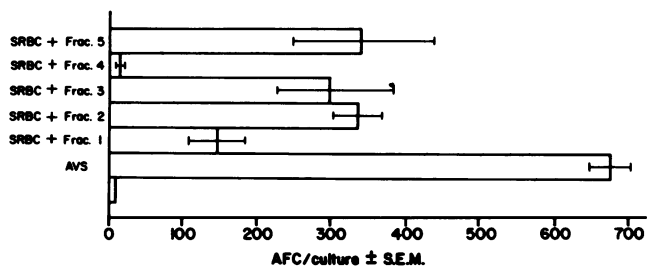


FIG. 4. *Effect of AVS and AVS fractions on the in vitro response of T-cell-deficient adherent mouse spleen cells to SRBC. Adherent mouse spleen cell monolayers were incubated with AVS (12.5 μg/culture) or AVS fractions (12.5 μg/culture), with or without SRBC. All of the cultures were assayed for anti-SRBC immunoglobulin M-secreting AFC after 4 days incubation. Results represent mean AFC per culture ± standard error of the mean for five experiments.*

was a carry-over of a strong mitogenic response or specificity of the blastogenic response to *A. viscosus* or both. It has been reported that human cord blood lymphocytes do not undergo blastogenesis in response to a water-soluble extract of *A. viscosus* (4, 27). Moreover, not all samples of human peripheral blood lymphocytes undergo blastogenesis in response to AVS. Burckhardt (5) has reported that although rat B lymphocytes respond nonspecifically to *A. viscosus*, rat T lymphocytes undergo stimulation with AVS only if these cells are from an animal that has been immunized previously with *A. viscosus*. These differences in the literature on the ability of AVS to induce DNA synthesis may be due, in part, to species differences and to the source of lymphocytes within the particular species.

It has previously been shown that normal adherent mouse spleen cells are incapable of significant antibody responses to the thymic-dependent antigen, SRBC, because of insufficient numbers of T-cells in these cell populations (20). The results we have obtained in this study have clearly demonstrated that a water-soluble extract of *A. viscosus* can augment the AFC response to SRBC of a population of T-cell-deficient spleen cells. There was an inverse dose-dependent relationship between the AVS concentration and the numbers of AFC per spleen cell culture; the numbers of AFC per culture decreased as the AVS concentration increased from 5 μg per culture to 25 μg per culture. At the same concentrations, the ability of AVS to induce a polyclonal response was minimal (Fig. 1). These results would suggest that AVS differs in its adjuvant properties from the LPS moiety of gram-negative bacteria.

The fractions that were obtained after fractionation of AVS on Biogel P-300 were tested for in vitro stimulation of DNA synthesis. Initially, fractions 3, 4, and 5 stimulated DNA synthesis on days 3 and 6 of incubation, whereas fractions 1 and 2 stimulated DNA synthesis on day 3 of incubation, but not on day 6. However, subsequent testing showed that fractions 1 and 2 also stimulated DNA synthesis on days 3 and 6 of incubation. The reason for this shift in activity is unknown.

The AVS fractions were also tested for their ability to augment an AFC response to SRBC. Fractions 1, 2, 3, and 5 were able to augment an AFC response to SRBC, but not to the degree of the unfractionated AVS. In contrast, fraction 4 was unable to augment the AFC response to SRBC.

Skidmore et al. (31) and Hoffman et al. (15) have reported that LPS has both mitogenic and adjuvant properties for the B lymphocyte. Hoffman et al. (15) have suggested that adjuvanticity and mitogenicity represent different pathways of B-cell activation by LPS and that these pathways are subject to different regulatory mechanisms. McGhee et al. (23) have shown that both T-cells and macrophages are required to obtain an augmentation of the AFC response to SRBC. Their report supports previous reports of Allison and Davies (1), Armerding and Katz (2), and Newburger et al. (26) that adjuvant properties of LPS are mediated by T-cells acting on B-cells.

It still is not clear whether the AVS is acting on a small number of residual T-cells that might be present in the adherent cell population but in insufficient numbers to activate B-cells to produce antibody to SRBC. Alternatively, the AVS may be acting directly on B-cells in the adherent cell population or as a T-cell-replacing factor in the cell culture system.

The water-soluble fractions and peptidoglycans of various bacterial cell walls have been shown to have adjuvant properties (8, 19, 33). The minimal structure required for adjuvant activity has been identified as a muramyl dipeptide which has been synthesized and used in place of the mycobacterial component in Freund complete adjuvant (3). More recently, Damais et al. (11) have reported that the synthetic muramyl dipeptide can stimulate DNA synthesis, as assessed by [$^3$H]Tdr incorporation. The biochemical analyses that were done on the AVS showed an enhancement of the AVS for amino acids and amino sugars that are commonly found in peptidoglycan. Reed (29) has shown that peptidoglycan is the major antigen of the *A. viscosus* cell wall. Since AVS contains small particulate cell wall fragments after centrifugation of the water-soluble extract at 10,000 × *g*, it is possible that the active component in the AFC assay is peptidoglycan. However, the ratio of glucosamine to muramic acid is not 1:1 in our preparations, indicating another source of glucosamine in the soluble AVS. It is also possible that one of the components active in the induction of DNA synthesis in this study is peptidoglycan.

It is of interest that several biological activities have been reported for another gram-positive bacteria, *Listeria monocytogenes*. Cell walls of *L. monocytogenes* have been reported to have adjuvant activity (7), to act as a B-cell mitogen (10), and to specifically induce resistance to subsequent infection (30). More recently, *L. monocytogenes* has been shown to contain an amphipathic moiety that is more similar to endotoxin than to lipoteichoic acid (36). Indeed, an amphipathic molecule from *A. viscosus* has recently been reported (37), and this molecule may (also)

be active in the induction of a blastogenic response.

We have shown that a water-soluble extract of *A. viscosus* can stimulate a blastogenic response to *A. viscosus* and augment an antibody response to SRBC. However, it is not clear what mechanism(s) and cell populations are involved. Studies to determine these questions are currently ongoing.

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