CYTOTOXIC REACTION OF HUMAN PNEUMOCYTES AND MONKEY KIDNEY CELLS AFTER EXPOSURE TO ORGANIC DUST AND DUST COMPONENTS

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Abstract

A colorimetric assay is used for quantitating cytotoxic effect on human pneumocytes (A549) and monkey kidney cells (VERO). The assay is conducted by incubating cell cultures with sterile extracts of cotton bract, suspension of pure endotoxin from *Enterobacter agglomerans* (LPS) or bacterial supernatant in microtiter plates for 2h and 24h. After exposure formazan is added, and 4h later solubilizer is added 16h prior to reading of the results in an ELIZA microplate reader at 570 nm. Absorption of formazan is directly related to viable cell number and their growth rate. Only crude cotton dust and extracts from *B. cereus* elicited an effect on the cell cultures. We find a similar response in the two cell lines. However, the monkey kidney cells are more susceptible to the extracts than human pneumocytes. After a few hours we find morphological changes in the cell monolayer without a cytotoxic reaction. After 24 hours we find a significant cytotoxic effect. No effect is seen after exposure to LPS, glucans, FMLP or tannin. LPS has no adjuvant effect in this assay when tested with crude cotton dust, tannin or *B. cereus*. The lack of reaction to compounds other than crude cotton and *B. cereus* indicates that the cytotoxic assay detects something different from the stimulation seen in macrophages with subsequent release of cytokines. It is not known if this cytotoxic reaction is involved in the pathogenesis of the respiratory symptoms observed among the exposed. However, we are working with other reactions to study the timing of the response after exposure to organic components.

Introduction

Exposure to organic dust is known to induce respiratory symptoms and disorders among workers in a wide range of occupations. An association has been found between the concentrations of Gram negative bacteria and the prevalence of byssinosis (1,2). Lipopolysaccharide (LPS) from cotton has been found to be associated with lung symptoms in many studies of cotton dust. This has been found in experimental (3-7) and epidemiologic studies (8,9). LPS has been shown to be associated with respiratory diseases among exposed workers in other occupations, i.e., farming and garbage sorting (10-14). In other organic dusts yet other components have been shown to be active in the causation of respiratory diseases among exposed workers (15-21).

Besides LPS, a wide range of other components in organic dusts may be active (20). Different substances have been found to be active in experimental studies including tannins (22-25) , *Bacillus spp* (26), N-Formyl-Methionyl-Leucyl-Phenylalanin (FMLP) (27) and glucans (28). Besides the direct effect of these compounds, some studies of LPS and other substances from organic dust have been indicative of an adjuvant effect of the concurrent exposure to different substances on the release of cytokines (29-31).

Studies of cell cultures have shown, that tannins from cotton dust inhibit phagocytosis, free radical production and induce the release of arachidonic acid from exposed endothelial cells (32). We have in previous studies of human pneumocytes shown a cytotoxic effect of *B. cereus* and other *bacilli* and also of crude cotton dust. However, we have not been able to demonstrate any effect of neither LPS alone nor with LPS as an adjuvant.

This study is undertaken to investigate the cytotoxic effect of organic dust and dust components including LPS from *Enterobacter agglomerans*, tannin, glucan, FMLP and *Bacillus spp*. supernatants in an in-vitro assay using human pneumocytes and monkey kidney cells. The cytotoxic potential is studied for each substance alone and with LPS as an adjuvant for cotton, glucan, *B. cereus* and tannin.

Materials and Methods

Materials:

LPS isolated from *Enterobacter agglomerans* was received from R.Rylander, Stockholm. Purified cotton bract tannin was received from Philip J. Bates, Thoracic Diseases Research Unit, Mayo Clinic. Beta-1-3 glucan (Curdland) from Wako Pure Chemical Ltd. lotnr: JPN9142 (032-09902), *Bacillus cereus* isolated from dust from a cotton mill in Vejle, Denmark and treated as described previously (33). N-Formyl-Methionin-Leucyl-Phenylalanin (Sigma Chemical, St. Louis, F-3506). All the components were made in a stock solution of 1 μg/ml in pyrogen free water, except FMLP which was diluted in 0.05M Na_2HPO_4 pH 7.4. All the components were used in dilutions from 0.1 pg/ml to 100 μg/ml. Crude cotton dust from a cotton mill in Vejle, Denmark, was extracted as described earlier (33).

Cell cultures:

A) Cultures of monkey kidney cells (VERO from American Type Culture Collection ATCC no: CCL 81 pass: 125-130) are grown in Earle's medium 199 (95%) with fetal bovine serum (5%, LPS content < 0.02 ng/ml; Gibco-BRL) and penicillin/streptomycin (100IU/100μg pr ml; JRH-Biosciences) at 37°C, 5% CO_2. For the assay $5x10^3$ cells/well are seeded.

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B) Cultures of human lung carcinoma cells (A 549 from American Type Culture Collection ATCC no: CCL 185 pass: 98-103) are grown in HAM's F 12K medium (90%) with fetal bovine serum (10%, LPS content < 0.02 ng/ml; Gibco-BRL) and penicillin/streptomycin (100IU/100μg pr ml; JRH-Biosciences) at 37°C, 5% CO_2. For the assay $4x10^3$ cells/well are seeded.

Cytotoxic assay:

CellTiter 96™ Non-Radioactive Cell Proliferation/Cytotoxicity assay (Promega) are used. Cells are grown in microtiter plates for cell cultures (Greiner) for two days. On day 3 the medium is exchanged with extracts in conc. 0.04-20.8 μg/well suspended in medium and incubated 2-24 hours. At this time the cells are nearly confluent and the medium is exchanged once again with medium containing dye (15% tetrazolium-salt). After another 4 hours of incubation solubilizer is added into the dye-medium and the suspension is left overnight at room temperature in a dark humid container. The results are read with a 570 nm messenger and 630 nm reference filter in an ELISA microplate reader (BIO-RAD 450), and plotted as percent of non exposed cells.

Statistics:

For significance t-testing and one-way analysis (SPSS statistical Package for the Social Science/IBM) is used. The measurement of 0.48 mg dust/well is the mean of 16 wells expressed as percent of untreated control cells (8 readings at concentration 0.32 mg dust/well and 8 readings at concentration 0.64 mg dust/well). The measurement of 15.6 mg dust/well is the mean of 16 wells expressed as percent of untreated control cells (8 readings at concentration 10.4 μg/well and 8 readings at concentration 20.8 μg/well).The measurement of 0.555 pg dust/well is the mean of 16 wells expressed as percent of untreated control cells (8 readings at concentration 10 pg dust/well and 8 readings at concentration 1 ng dust/well). The measurement of 55 mg dust/well is the mean of 16 wells expressed as percent of untreated control cells (8 readings at concentration 10 μg/well and 8 readings at concentration 100 μg/well).

Results

Figure 1 and 2 show the effects of crude cotton dust on human pneumocytes and monkey kidney cells after 24 hours of exposure. Although the effect in the two cell types is similar, the monkey kidney cells seems to be more susceptible to the extract than human pneumocytes. Figure 3 and 4 show the effects of extracts of *B. cereus* on the cell lines after 24 hours of exposure. Again the same pattern is seen with the monkey kidney cells as the most susceptible cell line.

From Table 1 it is seen that human pneumocytes reacts only with extracts from crude cotton dust and *B. cereus* in a dose dependent manner. This reaction is more pronounced after 24 hours of exposure than after 2 hours of exposure. However, when we study the cell monolayers in a phase contrast microscope after 2 hours of exposure, we do find morphological changes with rounding of the cells and lace-like appearance of the cell layer. No effect is seen after exposure to LPS, glucans, FMLP or tannin. LPS has no adjuvant effect in this assay when tested with crude cotton dust, tannin or *B. cereus*. The response towards the components with or without LPS is similar in shape of the curve as well as magnitude of response (Table 1 and Table 2).

Discussion

Earlier studies have shown that organic dusts elicit cytotoxic effects on cell cultures of human pneumocytes and monkey kidney cells (33,34). Components from the organic dusts have also been found to elicit a range of other effects on cell cultures, when they have been added to the medium. LPS induces the release of TNF-alpha from macrophages (35). Beta-1-3-D-glucans enhance the release of arachidonic acid in mice after LPS stimulation (31). Grifolan a specific ß-1-3-D-glucan induce the release of IL1, IL6 and TNF-alpha from a macrophage cell line (RAW264.7) in a dose dependent manner (28). Tannin induces the release of arachidone acid and IL1 from endothelial cells and macrophages(24,32). In this study we test the cytotoxic potential of cotton dust and dust components alone and in combination with LPS from *Enterobacter agglomerans*. We find only an effect after exposure to crude cotton dust and *B. cereus*. In this system we do not find any effect of the other compounds. We find a difference in the way human pneumocytes and monkey kidney cells react towards exposure. In monkey kidney cells there is a difference in the reaction to *B. cereus* and crude cotton dust, which is not seen in human pneumocytes. No effect is seen after two hours of exposure in the cytotoxic assay. However, in a qualitative assay we do see an effect of the exposure preceding the cytotoxic effect, like the one described by Johnson et al. for endothelial cells (36). The lack of reaction might not be surprising for FMLP, since earlier studies with this substance have found FMLP to be less potent then other substances in organic dust, ie LPS (27,37). The lack of reaction to the other compounds indicates that the cytotoxic assay detects something different from the stimulation seen in macrophages with subsequent release of cytokines (22-25). We are presently studying the cytokine release concurrently with the cytotoxic assay to see if there is an uncoupling of the two response parameters.

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References

1. Rylander, R. and M. Lundholm. 1978. Bacterial contamination of cotton and cotton dust and effects on the lung. Br J Ind Med. 35:204-207.

2. Lundholm, M. 1981. Airborne gram-negative bacteria in cotton mills and sewage plants. University. of. Gothenburg. Gothenburg.

3. Rylander, R. and B. Bake. 1987. Airway hyperreactivity and bronchoconstriction after inhalation of cell bound endotoxin. Am Rev Respir Dis. 135:a234.

4. Demaria, T. F. and R. Burrell. 1980. Effects of inhaled endotoxin-containing bacteria. Enviromental Research. 23:87-97.

5. Rylander, R., P. Haglind, and M. Lundholm. 1985. Endotoxin in cotton dust and respiratory funtion decrement among cotton workers in an experimental cardroom. Am Rev Respir Dis. 131:209-213.

6. Rylander, R., I. Mattsby, and M. C. Snella. 1980. Airway immune response after exposure to inhaled endotoxin. Bull. Europ. Physiopath. Resp. 16:501-509.

7. Castellan, R. M., S. A. Olenchock, K. B. Kinsley, and J. L. Hankinson. 1987. Inhaled endotoxin and decreased spirometric values. The New England Journal of Medicine. 317:605-610.

8. Kennedy, S. M., D. C. Christiani, E. A. Eisen, D. H. Wegman, I. A. Greaves, S. A. Olenchock, T. Ye, and P. Lu. 1987. Cotton dust and endotoxin exposure-response relationships in cotton textile workers. Am Rev Respir Dis. 135:194-200.

9. Sigsgaard, T., O. F. Pedersen, S. Juul, and S. Gravesen. 1992. Respiratory disorders and atopy in cotton wool and other textile mill workers in Denmark. Am J Ind Med. 22:163-184.

10. Sigsgaard, T., P. Malmros, L. Nersting, and C. Pedersen. 1994. Work related symptoms and lung function measurements among Danish refuse workers. Am. Rev. Respir. Dis. 149:1407-1412.

11. Sigsgaard, T., B. Bach, and P. Malmros. 1990. Respiratory impairment among workers in a garbage-handling plant. Am J Ind Med. 1:92-93.

12. Sigsgaard, T., A. Abell, and L. D. Jensen. 1994. Work related symptoms and lung function measurements in paper mill workers exposed to recycled water. Occ. Hyg. 1:177-189.

13. Hollander, A., D. Heederik, and H. Kauffman. 1994. Acute respiratory effects in the potato processing industry due to a bioaerosol exposure. Occup Environ Med. 51:73-78.

14. Sherson, D., I. Hansen, and T. Sigsgaard. 1989. Occupationally related respiratory symptoms in trout-processing workers. Allergy. 44:336-341.

15. Larsson, K. A., A. G. Eklund, L-O. Hansson, B-M. Isaksson, and P. O. Malmberg. 1994. Swine dust causes intensive airways inflammation in healthy subjects. Am J Respir Crit Care Med. 150:973-977.

16. Malmberg, P. and K. A. Larsson. 1993. Acute exposure to swine dust causes bronchial hyperresponsiveness in healthy subjects. Eur. Respir. J. 6:400-404.

17. Manfreda, J., V. Holford-Strevens, M. Cheang, and C. P. W. Warren. 1986. Acute symptoms following exposure to grain dust in farming. Environmental Health Perspectives. 66:73-80.

18. Donham, K. J., S. J. Reynolds, P. Whitten, J. Merchant, L. Burmeister, and W. J. Popendorf. 1995. Respiratory dysfunction in swine production facility workers: Dose-response relationships of environmental exposures and pulmonary function. Am J Ind Med. 27:405-418.

19. Rask-Andersen, A. 1988. Pulmonary reactions to inhalation of mould dust in farmers with special reference to fever and allergic alveolitis;Thesis. Universitatis Uppsaliensis, Uppsala. 1 pp.

20. Rask-Andersen, A., P. Malmberg, and M. Lundholm. 1989. Endotoxin levels in farming: absence of symptoms despite high exposure levels. Br J Ind Med. 46:412-416.

21. Chan-Yeung, M., S. Vedal, J. Kus, L. McLean, D. A. Enarson, and K. S. Tse. 1984. Symptoms, Pulmonary function, and bronchial hyperreactivity in western red cedar workers compared with those in office workers. Am. Rev. Respir. Dis. 40:53-57.

22. Rohrbach, M. S., R. A. Rolstad, and J. A. Russell. 1986. Tannin is the Major Agent Present in Cotton Mill Dust Responsible for Human Platelet 5-Hydroxytryptamine Secretion and Thromboxane Formation. Lung. 164:187-197.

23. Kreofsky, T. J., J. A. Russell, and M. S. Rohrbach. 1990. Inhibition of alveolar macrophage spreading and phagocytosis by cotton bract tannin. A potential mechanism in the pathogenesis of byssinosis. Am. J. Pathol. 137:263-274.

24. Cloutier, M. M. and M. S. Rohrbach. 1986. Effects of endotoxin and tannin isolated from cotton bracts on the airway epithelium. Am Rev Respir Dis. 134:1158-1162.

25. Johnsen, C. M., M. N. Hanson, and M. S. Rohrbach. 1986. Toxicity to endothelial cells mediated by cotton bract tannin. Am J Pathol. 122:399-409.

26. Hoult, B. and A. F. Tuxford. 1991. Toxin production by Bacillus pumilus. J Clin Path. 44:455-458.

27. Burell, R. and R. Rylander. 1985. Comparative toxicity studies between bacterial lipopolysaccharide (endotoxin) and N-formyl methionyl peptide as factors in the pathogenesis of byssinosis. J. Toxicol. Environ. Health. 16:607-613.

28. Adachi, Y., M. Okazaki, N. Ohno, and T. Yadomae. 1994. Enhancement of cytokine production by macrophages stimulated with (1-3)beta-D-glucan, grifolan, isolated from grifola frondosa. Biol. Pharm. Bull. 17:1554-1560.

29. Norn, S., L. Bæk, C. Jensen, P. S. Skov, H. Permin, J. O. Jarl¢v, and C. Koch. 1986. Influence of bacterial endotoxins on basophil histamine release. Allergy. 41:125-130.

30. Clementsen, P., N. Milman, M. Kilian, A. Fomsgaard, L. Baek, and S. Norn. 1990. Endotoxin from Haemophilus influenzae enhances IgE-mediated and non-immunological histamine release. Allergy. 45:10-17.

31. Ohno, N., N. Asada, Y. Adachi, and T. Yadomae. 1995. Enhancement of LPS triggered TNF-alpha production by 1-3-beta-D-glucans in mice. Biol. Pharm. Bull. 18:126-133.

32. Rohrbach, M. S., T. J. Kreofsky, Z. Vuk-Pavloviz', and D. Lauque. 1992. Cotton condensed tannin: a potent modulator of alveolar macrophage host-defence function. Basic Life Sci. 59:803-824.

33. Sigsgaard, T., V. Roepstorff, A. F. Tuxford, and B. Hoult. 1993. Cytotoxicity of Bacillus species to Vero cells. In Proceedings - XVIIth Cotton Dust Research Conference. R.R. Jacobs, L.N. Domelsmith, and P.J. Wakelyn, editors. Cotton Incoorporated, Memphis. 258-261.

34. Roepstorff, V. and T. Sigsgaard. 1995. Cytotoxic effect of extracts fron tannin, treated and untreated cotton on human pneumocytes. In Proceedings - XVIIIth cotton dust research conference. R.R. Jacobs and R. Rylander, editors. Cotton Incoorporated, Memphis. 272-275.

35. Rietschel, E. T. and H. Brade. 1992. Bacterial Endotoxins - An integral part of many bacteria, these molecules are at once brutal beneficial to humans. Efforts are under way to block the bad effects and harness the good. Scientific American. 26-33.

36. Johnson, C. M., M. N. Hanson, and M. S. Rohrbach. 1986. Endothelial cell cytotoxicity of cotton bracts tannin and aqueous bracts: Tannin is the predominant cytotoxin present in aqueous cotton bracts extract. Environmental Health Perspectives. 66:97-104.

37. Frazer, D. G., V. A. Robinson, K. C. Weber, W. Jones, P. D. Siegel, M. W. Barger, B. Masters, C. A. Chandler, D. Vincent, and V. Castranova. 1992. Pulmonary response of the guinea pig animal model to n-Formyl-Methionyl-Leucyl-Phenylalanine (FMLP) liquid aerosol. In Proceedings - XVIIIth Cotton Dust Research Conference. R.R. Jacobs, P.J. Wakelyn, and R. Rylander, editors. Cotton Incoorporated, Memphis TN. 266-270.

Table 1. Human pneumocytes exposed to extracts of different dusts for 2 and 24 hours. Each well is the mean of 16 readings and are reported as percentage of the unexposed well.

Compound	2 hours		24 hours	
	concentration*		concentration*	
	I	II	I	II
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Crude cotton §§	100(3)^{B}	113(3)$^{-,E}$	104(3)^{E}	53(6)$^{-,A}$
B. cereus§§	104(4)^{C}	90(5)$^{-,A}$	65(4)^{A}	54(2)$^{-,A}$
Crude cotton + LPS §§	92(3)^{A}	105(5)$^{-,C,D}$	101(4)^{D,E}	58(9)$^{-,A}$
B. cereus + LPS §§	99(7)^{B}	87(5)$^{-,A}$	70(7)^{A}	57(5)$^{-,A}$
Tannin + LPS§	102(3)^{C}	103(4)^{B,C,D}	107(10)^{E}	108(11)^{D}
LPS §	104(12)^{C}	113(5)$^{-,B}$	91(2)^{B,C}	91(4)^{B,C}
FMLP §	89(7)^{A}	99(5)$^{-,B}$	95(3)^{C,D}	95(3)^{C}
Tannin §	94(4)^{A,B}	106(6)$^{-,D}$	89(2)^{B}	99(2)$^{-,C}$
Curdlan §	92(5)^{A,B}	101(4)$^{-,B,C}$	113(8)^{E}	107(10)$^{-,D}$

_: $p < 0.05$ t-test
*: cells in column with the same letter are not significantly different ($p < 0.05$)
I: if § = 555 pg/ml if §§ = 4.6 μg/ml
II: if § = 55 μg/ml if §§ = 208 μg/ml

Table 2. Monkey kidney cells exposed to extracts of different dusts for 2 and 24 hours. Each well is the mean of 16 readings and are reported as percentage of the unexposed well.

Compound	2 hours		24 hours	
	concentration		concentration	
	I	II	I	II
	Mean (SD)*	Mean (SD)	Mean (SD)	Mean (SD)
Crude cotton§§	94(3)^{C}	103(8)^{C}	74(4)^{A,B}	13(5)$^{-,A}$
B. cereus§§	84(4)^{A}	77(5)$^{-,A}$	76(7)^{B}	48(4)$^{-,B}$
Crude cotton + LPS§§	99(6)^{D}	76(6)$^{-,A}$	86(3)^{C}	14(1)$^{-,A}$
B. cereus + LPS§§	92(7)^{B}	82(5)$^{-,A,B}$	69(9)^{A}	49(6)$^{-,B}$
Tannin + LPS§	100(10)^{D}	110(5)$^{-,D}$	94(6)^{D}	97(7)^{D}
LPS§	99(4)^{D}	110(3)^{D}	80(42)^{B,C}	95(3)$^{-,D}$
FMLP§	87(1)^{A,B}	98(2)$^{-,C}$	84(3)^{C}	94(5)$^{-,D}$
Tannin§	89(6)^{B}	98(10)$^{-,C}$	88(9)^{D}	100(15)$^{-,D}$
Curdlan§	95(4)^{D}	87(5)$^{-,B}$	81(4)^{C}	83(9)^{C}

_: $p < 0.05$ t-test
*: cells in column with the same letter are not significantly different ($p < 0.05$)
I: if § = 555 pg/ml if §§ = 4.6 μg/ml
II: if § = 55 μg/ml if §§ = 208 μg/ml

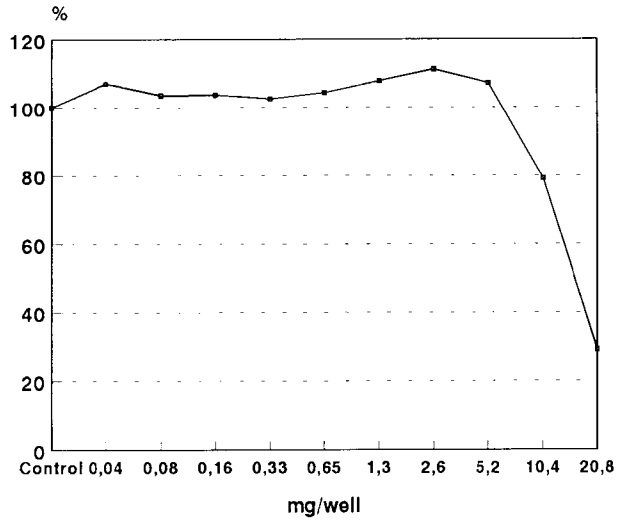

Figure 1. ELISA reading of human pneumocytes cells exposed to extracts of crude cotton dust for 24 hours.

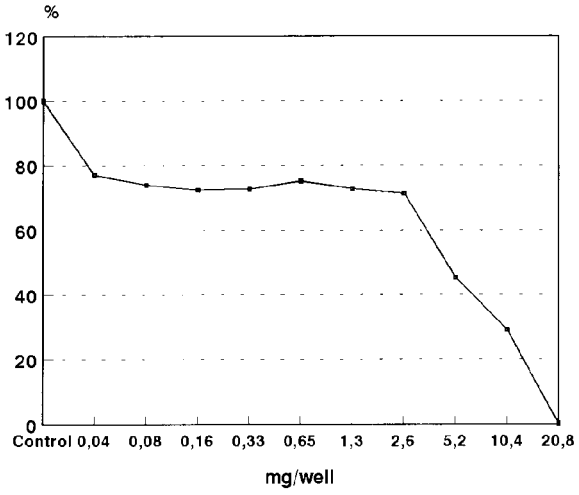

Figure 2 . ELISA reading of monkey kidney cells exposed to extracts of crude cotton dust for 24 hours.

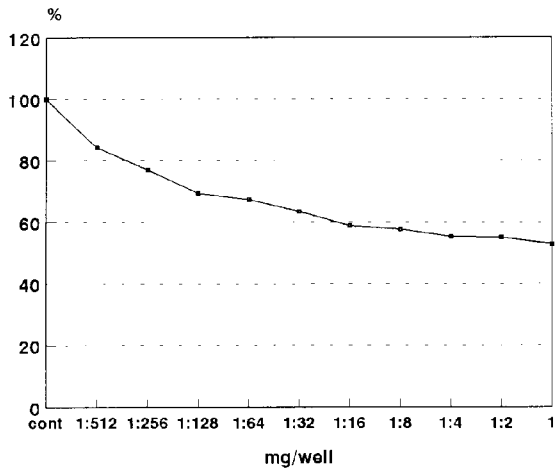

Figure 3. ELISA reading of human pneumocytes exposed to supenatant from *B. cereus* for 24 hours.

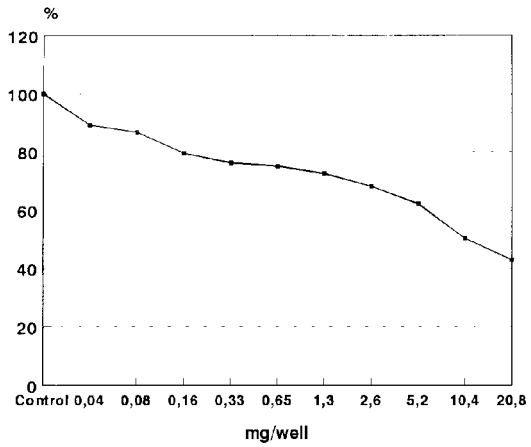

Figure 4. ELISA reading of monkey kidney cells exposed to supenatant from *B. cereus* for 24 hours.