THE BACTERICIDAL POWER OF SERUM FOR MORAXELLA IN PYRIDOXINE DEFICIENCY

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The remarkable decline in Moraxella infections is believed to be the result of improved nutrition. Pyridoxine is the nutritional component thought to be specifically associated with Moraxella angular conjuntivitis. [1,2].

Angular conjunctivitis is a surface infection in which phagocytosis, intracellular digeston and serum bactericidal action might be expected to play an important role in the first line of defense. It was found in in-vitro experiments that phagocytosis[3] and also the digestive capacity of phagocytes[4] were impaired in pyridoxine deficiency.

Agnew and Cook[5] in 1949, Wertman and Sarandria[6] in 1951, Axelrod, Hopper and Long[7] in 1961 and Axelrod and Trakatellis[8] in 1964 found that pyridoxine impaired the synthesis of circulating antibodies. Pruzansky and Akelrod[9] believed that the synthesis of complement was not affected by pyridoxine deficiency. Fleming[10] in 1922, Voss[11] in 1964 and Glynn and Milne[12] in 1965 found lysozyme alone or in combination with antibody and complement able to lyse or kill a variety of bacteria including gram-negative rods. Glynn and Milne[13] in 1967 postulated the existence of a fourth bactericidal factor in the serum that could be absorbed by bentonite.

A significant difference was found between the sera of pyridoxine deficient and control animals with regard to conditioning Moraxella for digestion and possibly also for ingestion by phagocytes.

Nothing is known, however, about the direct bactericidal action of pyridoxine deficient and control sera or their components on Moraxella and this was, therefore, studied.

MATERIALS AND METHODS

The diet is given in a previous report[14]. Guinea-pigs of 350 gm. were used throughout the experiments. Moraxella nonliquefaciens strain 21 (Mor. 21 N. L.) was used as a test strain.

Serum: Blood obtained by cardiac puncture was allowed to clot on ice. Separated serum within each group was pooled and freezed at —20°C in aliquots of 2 ml until used for the analysis of the bactericidal capacity

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of the serum components. In those tests in which the bactericidal ability of the whole serum was compared, the sera of the diet and control animals were used immediately after the clot was separated.

Antibody: The indirect fluorescence technique was used for a semi-quantative estimate of the serum anti-Mor. 21 N. L. antibodies. A total amount of 3,6 ml of a 2% guinea-pig γ globulin solution, isolated in a routine fashion, was injected intraperitoneally in the rabbit on 4 successive days per week during 3 successive week. The anti-guinea-pig γ globulin — rabbit γ globudin was conjugated to fluorescein-isothiocyanate in such a way that the conjugate contained ½% rabbit γ globulin in final concentration. The level of guinea-pig antibody directed against M. nonliquefaciens was expressed by grading the intensity of fluorescence from 0 — 5, The average of 5 observations was used.

Absorption of antibodies: Essentially the method of Wardlaw [15] was followed. Mor. 21 N.L. used for absorption was harvested from the Moraxella base mediaum [16] after 48 hours of incubation and washed three times in Hanks-Tris solution buffered at pH 7,4. A portion of the suspension was dried on a planchette to determine the dry weight of these bacteria per volume unit. A volume of the suspension corresponding to 50 mg dry weight of bacteria for each ml serum to be absorbed was centrifuged at 36.500 x g and after discarding the supernatant the bacterial pellet was suspended in the serum and the mixture stirred during 15 minutes. Serum and bacterial suspension were kept at 0°C and the absorption was carried out on ice. This treatment was repeated until the antibodies were removed. These sera were coded: AB —.

Assay of lysozyme in serum: The lysozyme content was determined with an agar diffusion method developed after the principles of Fleming 1922[10], Goldsworthy and Florey 1930[17], Boasson 1938[18] and Smolelis and Hartsell 1949[19]. Ultraviolet killed and freeze-dried M. lysodeikticus was used as a substrate. The cells were suspended in 1% agarose in 0,067 M phosphate buffer of pH 6,2 in a final concentration of 50 mg per 100 ml medium. The substrate was poured to a thickness of 1 mm on glasslides. Whatman No. 3 filterpaperdisks of 6 mm diameter carrying 5 cmm serum were placed on the substrate. After 24 hours of incubation at 37°C the slides with agar were dried, fixed and stained according to the technique of van Arkel, Ballieux and Jordan 1963[20]. The diatemer lysis was compared to the diameter lysis obtained using cristalline egg-white lysozyme (Fluka Laboratories) as standard.

Inactivation of serum lysozyme: Antilysozyme antibody and bentonite absorption were used for the inactivation of serum lysozyme. Rabbit anti egg-white lysozyme antibodies were obtained by vaccination with a total of 90 mg aluminum phosphate absorbed egg-white lysozyme. Bentonite absorption of the serum was done essentially according to the technique of Myrvik and Weiser[21]. 1 gm of bentonite was suspended in 500 ml distilled water and washed by cycles of centrifugation and resuspension until the pH initially around 13,0 had fallen to 7,7. Finally the sediment

was resuspended in 100 ml distilled water and after settling and discarding the coarse bentonite particles, a volume of the fine particle suspension corresponding to 5 mg of dry weight of bentonite for every ml serum to be absorbed was concentrated and the pellet mixed with serum. Absorption was carried out at 0°C during 15 minutes. Serum and bentonite were separated by centrifugation at 36.500 x g and the pellet discarded.

The agar diffusion technique was used to test for residual lysozyme in serum. Serum absorbed with bentonite was coded L-, BAF-. In those combinations in which only the effect of the bentonite absorbable factor was the object of study, the absorbed serum lysozyme was reconstituted whith egg-white lysozyme in a final concentration of 0,061 mg per ml. These sera were coded L +, BAF —. By absorbing and reconstituting various serum components, the bactericidal capacity of 12 combinations on Mor. 21 N. L. was examined.

The bactericidal capacity of the serum components: Mor. 21 N. L., washed three times in Hanks-Tris solution, was suspended in this medium in a final concentration of 1,55 x 10^6 cells per ml. To this system whole or treated sera were added to a final concentration of 20%. In the control suspension 100 mg% bovine albumin (Armour fraction V) was used instead of serum. The bacteria-serum suspension and the controls were placed in siliconized screwcap bottles on a turntable inclined at 45° making 6 R.P.M. and incubated at 37°C for 1 hour. Viable counts were done according to the technique of Miles and Misra[22]. The bactericidal power of the systems was expressed as reduction factor (R.F.), i.e. the ratio of bacteria recovered in the control systems with bovine albumin and from the systems with pyridoxine deficient or normal, whole or treated guinea-pig serum.

The bactericidal capacity of the whole serum: Paired observations on the bactericidal capacity of the whole serum of pyridoxine deficient and normal guinea-pigs was carried out as described above.

Serum of 9 pairs of randomly selected diet and control animals were added to the bacterial suspensions in a final concentration of 50 — 25 — 12,5 — 6,25 — 3,13 and 1,56%. Results were analyzed by using the non-parametrical test of Walsh.

RESULTS

Antibody titer in the pooled serum: The titer of antibodies directed against Mor. 21 N. L. measured with the fluorescent antibody technique was 1:8. Two absorption cycles were sufficient to remove all antibody from the serum. Complement was fixed by the absorption of antibodies in spite of absorption at 0°C. The haemolytic titer decreased from 1: 40 to 1:5. The loss of complement was symbolized as C ± in the table.

Lysozyme concentration in the pooled serum: The average diameter lysozyme lysis in the pooled serum was 14,92 mm (95% confidence interval: 14, 44-15, 40). In Figure 1 the diameter of lysozyme lysis as function

of the concentration of the guinea- pig pooled serum was equivalent to 0,061 mg egg-white lysozyme per ml. This was thus the concentration that was reconstituted for the L+ combinations. One cycle of bentonite absorption removed all lysozyme from the guinea-pig serum. The haemolytic titer of complement after bentonite absorption decreased from 1:40 to 1:35.

The bactericidal power of the whole serum: The reduction factors of the diet and control sera are given in Table 1. No difference was found in bactericidal capacity of the sera of pyridoxine deficient and control animals at anly level of serum concentrations used. With a serum concentration of 50% an average of 75% of the Moraxella population was reduced (R. F. 3,98). With a final serum concentration of 25% more than half of the bacterial cells were killed. With a final concentration of the sera below 12,5% no reduction was found. The total average reduction and percent reductions as well as the 95% confidence intervals for the various concentrations are found also in Table 1.

The bactericidal power of the treated sera: The magnitude of killing expressed as reduction factors by the various combinations of serum components are shown in Table 2. The reduction of Moraxella by the untreated serum was 30%. Significant reduction of bacteria was found only in those sera in which complement was present. The bactericidal activity of complement alone could not be studied since with antibody absorption complement was fixed.

For Mor 21 N. L. it was found that lysozyme alone had no bactericidal effect. Lysozyme in the combination with complement and antibody did not have a greater bactericidal capacity than antibody and complement alone.

BAF had the greatest bactericidal capacity of the serum for Mor. 21 N. L. in the presence of complement, antibody and lysozyme. In all other combinations in which the bentonite absorbable factor was present no significant reduction was found. In some of the sera the reduction factor was smaller than one. This was probably as a result of some cell divisions of some cells during the incubation period.

The reduction factor of the combination of C ±, Ab —, L +, BAF + fluctuated widely and significantly between various trials. No difference was found, however, between the pairs of diet and control sera. In the table the average R. F. of all trials with the C ±, Ab —, L +, BAF + combination are given.

While the various treated sera had different bactericidal capacity, no difference between identical combinations of pyridoxine deficient and control sera was found.

DISCUSSION

Although high rabbit serum antibody titers could be obtained against egg-white lysozyme, this antiserum could not bind the guinea pig serum lysozyme, presumably because of antigenic differences in egg-white and guinea-pig serum lysozyme. Therefore, the bactericidal capacity of the se-

ra with the L —, BAF +, complement and antibody combinations could not be studied.

In conjuctival smears from patients with angular conjunctivitis Moraxella are seldomly seen in the polymorphonuclear leucocyte. If pyridoxine deficiency would be the most important factor in the etiology of the disease, the mechanism could be that of depressing the phagocytic activity. It was found in an in-vitro model system using a suitable combination of parameters that there was a highly significant difference in phagocytosis, but modest in amount between pyridoxine deficient and normal guinea-pig phagocytes for each of the main cell types as well as for all the cells participating in phagocytosis. By increasing the bacterial load, the failure of the pyridoxine deficient phagocytes to meet the challenge both in ingestion of bacteria as well as in mobilizing an increased number of cells for phagocytosis became very apparent[3].

The ability of phagocytes to digest the organisms is even more important than phagocytosis. In in-vitro phagocyte-homologous serum systems of pyridoxine deficient and control guinea-pigs it appeared that the all over digestive capacity of the control phagocytes was much better. The difference in digestion of the pyridoxine and control phagocytes alone, however, was in rate rather than in amount and the slower digestive ability of the pyridoxine deficient phagocytes was correlated with a decrease of the intraphagocytic enzyme myeloperoxidase[4]. Consequently, the role of serum in the elimination of bacteria seemed important.

Glynn and Milne (1967) felt that the bactericidal capacity of serum for E. coli was closely dependant on the complement concentration. They found the antibody concentration for optimal bacterial killing so small, that they considered the antibody concentration under normal conditions not a limiting factor.

Lysozyme played also a role in the killing of bacteria and lysis of E. coli. Addition of lysozyme to the bentonite absorbed serum did not recontitute the killing ability of the serum. On the basis of these findings they concluded that a bentonite absorbable factor was present in the serum. From our results this appeared very likely.

We found that serum nor the serum components of pyridoxine deficient and control guinea-pigs differed in their ability to kill Moraxella. In conditioning the bacteria, however, for subsequent digestion and possibly also for ingestion there was a significant difference between sera from pyridoxine deficient and control animals.

RESUMO

A conjuntivite angular é uma infecção superficial. A ação bactericida do soro é um dos elementos de primeira linha de defesa na conjuntivite angular. Acredita-se que o declínio das infecções por Moraxella seria o resultado de uma melhor nutrição, sendo a deficiência de piridoxina o elemento específico. Não existem estudos científicos específicos que comprovem ou não a importância da piridoxina sobre a ação bactericida do soro. Os autores fazem a descrição do material e método utilizados para a observação da atividade bactericida do soro, utilizando animais de laboratório.

Os resultados mostram uma notável ação bactericida do soro para Moraxella. Essa ação desaparece inativando-se o complemento e o anticorpo. No entanto o poder bactericida do soro não foi alterado na deficiência de piridoxina.

SUMMARY

Serum has a marked bactericidal activity for Moraxella. This action disappears after complement inactivation and after antibody absorption. Complement and possibly also antibody are necessary components for the serum bactericidal capacity. The bentonite absorbable factor was also an important bactericidal component, in the presence of complement and antibody. Moraxella was not lysed by lysozyme alone nor in the combination of lysozyme and complement or antibody.

Although there was a significant difference in indirect effect between pyridoxine deficient and control sera with regard to conditioning Moraxella for digestion and possibly also for phagocytosis a difference between pyridoxine deficient and control sera or their components with regard to direct bactericidal power was not found.

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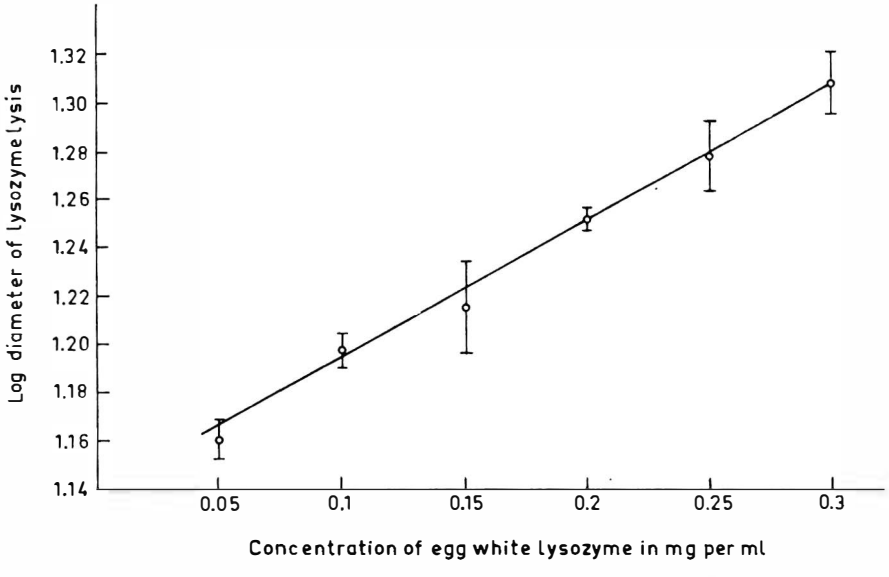

FIG. 1 — The logarithm of the diameter lysozyme lysis of **M lysodeikticus** substrate as function of the concentration of egg-white lysozyme.

TABLE 1 — The killing of M. nonliquefacions strain 21 by sera of pyridoxine deficient and control guinea-pigs expressed as reduction factor.

N.°	final concentration of the sera											
	50%		25%		12,5%		6,25%		3,13%		1,56%	
	diet	control	diet	control	diet	control	diet	control	diet	control	diet	control
1	2,69	2,74	1,03	1,07	1,19	1,45	0,91	0,57	0,74	0,81	1,24	1,16
2	3,97	7,06	2,86	7,06	7,50	4,00	2,00	2,00	2,40	2,40	2,40	1,71
3	1,44	2,64	2,32	2,57	1,09	1,78	0,60	1,16	0,59	0,63	0,69	0,78
4	2,46	4,83	1,66	2,68	2,20	1,77	2,75	3,09	2,04	2,51	2,25	1,45
5	10,98	3,04	2,49	1,96	1,51	1,50	1,20	1,08	1,07	1,05	0,98	1,34
6	4,86	1,70	2,12	1,08	1,55	1,10	1,53	0,83	0,78	0,95	1,68	1,02
7	2,62	1,75	1,48	1,28	0,42	0,41	0,31	0,35	1,15	0,82	0,29	0,34
8	6,23	7,79	2,84	3,66	1,96	2,00	2,02	2,16	1,39	1,37	1,21	1,28
9	2,58	2,18	1,32	1,13	1,23	1,40	0,97	0,95	0,93	0,81	0,83	1,26
TA	4,20	3,75	2,01	2,50	2,07	1,71	1,37	1,35	1,23	1,26	1,29	1,15
TA DC/CI	3,98	(2,77;5,19)	2,26	(1,59;2,93)	1,89	(1,14;2,64)	1,36	(0,98;1,74)	1,25	(0,95;1,55)	1,22	(0,96;1,48)
% red.	75		56		47		26		20		18	

TA = Total average
TA DC/CI = Combined total average of diet and control systems and 95% confidence interval
% red. = percent reduction

Table 2 — The killing of M. nonliquefaciens strain 21 by serum and serum components expressed as reduction factors and the 95% confidence intervals

	serum components	reduction factor	95% confidence interval
1	C+, Ab+, L+, BAF+	1,43	1,23 — 1,63
2	C+, Ab+, L+, BAF—	1,06	0,93 — 1,19
3	C+, Ab+, L—, BAF—	1,18	1,03 — 1,33
4	C±, Ab—, L+, BAF+	0,98	0,87 — 1,09
5	C±, Ab—, L+, BAF—	0,87	0,77 — 0,97
6	C±, Ab—, L—, BAF—	0,76	0,67 — 0,85
7	C—, Ab+, L+, BAF—	0,96	0,84 — 1,08
8	C—, Ab+, L—, BAF—	0,89	0,78 — 1,00
9	C—, Ab+, L+, BAF+	0,86	0,76 — 0,96
10	C—, Ab—, L+, BAF—	0,79	0,70 — 0,88
11	C—, Ab—, L—, BAF—	1,00	0,87 — 1,13
12	C—, Ab—, L+, BAF—	0,94	0,83 — 1,05

C = complement; Ab = antibody; L = lysozyme; BAF = bentonite absorbable serum-factor; + = present; — = absent.
The haemolytic titer of complement was 1:40; C ± = haemolytic titer of complement of 1:5.

REFERENCES

1. MITSUI, Y., HINOKUMA, S. and TANAKA, C. — Etiology of angular conjunctivitis. Am. J. Ophth. 34:1579, 1951.

2. IRINODA, K. and MIKAMI, H. — Angular blepharo-conjunctivitis and pyridoxine (vitamin B6) deficiency. Arch. Ophth. 60:303, 1958.

3. VAN BIJSTERVELD, O. P. — In-vitro phagocytosis in pyridoxine deficiency. J. Med. Microbiol. 4:165, 1971.

4. VAN BIJSTERVELD, O. P. — The digestive capacity of pyridoxine deficient phagocytes in-vitro. J. Med. Microbiol. 4:337, 1971.

5. AGNEW, L. R. C. and COOK, R. — Antibody production in pyridoxine-deficient rats. Brit. J. Nutr. 2:321, 1949.

6. WERTMAN, K. and SARANDRIA, J. L. — Complement-fixing murine typhus antibodies in vitamin deficiency states. Proc. Soc. Exp. Biol. Med. 76:388, 1951.

7. AXELROD, A. E., HOPPER, S. and LONG, D. A. — Effects of pyridoxine upon circulating antibody formation and skin hypersensitivity reactions to diphtheria toxoid in guinea-pigs. J. Nutr. 74:58, 1961.

8. AXELROD, A. E. and TRAKATELLIS, A. C. — Relationship of pyridoxine to immunological phenomena. Vitamins Hormones, 22:591, 1964.

9. PRUZANSKY, J. and AXELROD, A. E. — Effect of B-complex deficiencies on rat serum complement. Proc. Soc. Exp. Biol. Med. 88:179, 1955.

10. FLEMING, A. — On a remarkable bacteriolytic element found in tissue and secretions. Proc. Roy. Soc. B. 93:306, 1922.

11. VOSS, J. G. — Lysozyme lysis of gram-negative bacteria without production of spheroplasts. J. Gen. Microbiol. 35:313. 1964.

12. GLYNN, A. A. and MILNE, C. M. — Lysozyme and immune bacteriolysis. Nature 207:1309, 1965.

13. GLYNN, A. A. and MILNE, C. M. — A kinetic study of the bacteriolytic and bactericidal action of human serum. Immunology 12:639, 1967.

14. VAN BIJSTERVELD, O. P. — The conjunctival flora in pyridoxine deficiency. E. E. N. T. 50:444, 1971.

15. WARDLAW, A. C. — The complement-dependent bacteriolytic activity of normal human serum. I. The effect of pH and ionic strength and the role of lysozyme. J. Exp. Med. 115:1231, 1962.