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STUDIES ON THE EFFECTS OF PENICILLIN AND STREPTOMYCIN ON VACCINE LYMPH (CALF LYMPH) UNDER DIFFERENT CONDITIONS

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With the best of care, heavy bacterial contamination of vaccine lymph (calf lymph) is both incidental and inevitable during its preparation. The bacterial flora from the surroundings, the excreta and the skin of the animals are so varied and numerous that the vaccine lymph may contain as much as even 500 million organisms per ml., particularly in the tropics. Though the organisms belong mostly to the cocoi group, a few B.subtilis, B.coli, pseudomonas, yeasts and fungi are or may also be present. Annerobio organisms may also be occasional contaminants. None of the methods advocated so far to effect bacterial reduction of the vaccine lymph can be considered satisfactory. While some workers have obtained bacteria-free vaccine from sources other than calves, like egg and tissue culture, the use of the same has not met with universal approval for reasons of uncertain immunity. From point of protection, calf lymph has stood the test of time. Many Institutes in India employ what is known as "chloroform process" for the bacterial purification of vaccine lymph. In this method chloroform vapour is bubbled through the vaccine lymph for a known duration. But as chloroform has no action on spores, the spore-bearers present in the lymph persist even after chloroforming. Other countries use different methods. But no method so far has been found to give absolute bacterial sterility of vaccine lymph consistently.

The antibiotics by virtue of their action on bacteria should be of great value in the purification of vaccine lymph and some work has already been done, using penicillin for the purpose.

A detailed study on the effects of penicillin and streptomycin on vaccine lymph under different conditions has been made by the author and the results are discussed in this paper.

I. EFFECTS OF PENICILLIN ON GLYCERINATED VACCINE LYMPH

Effect of penicillin on glycerinated vaccine lymph has already been studied by some workers. While most workers [1,2,3] report spectacularly encouraging results with penicillin, the author's [4] experience of the same has been disappointing. It may be stressed at the outset that the use of such penicillin-treated lymphs is fraught with grave risks as penicillin not only does not bring about any bacterial reduction, but acting as a mask gives extremely deceptive results. These workers seem to have ignored the interference of the residual penicillin of penicillin-treated lymphs in the tests.

In one of the experiments, penicillin was used in concentrations varying from 500 to 2000 units per ml of glycerinated vaccine lymph (Table I). The sterility test by the usual pourplate method showed all the samples to be sterile within 24 hours' contact with penicillin. While one sample was deliberately kept at room temperature (Bangalore about 23° C), all others were preserved in the cold storage. When the sterility test was repeated after an interval of one month, the sample kept at room temperature showed numerous staphylococcus and a few B. subtilis group of organisms, while the samples kept in the cold storage gave no growth whatever. At this stage the samples were also tested for the presence of penicillin. While the samples kept in the cold storage showed no appreciable loss of penicillin, the one kept at room temperature revealed almost complete absence of penicillin.

TABLE I

No.	Penicillin conc. (units per ml.)	Preser. Temp.	colony count on vaccine lymph after				
			24 hrs.	2 days	3 days	1 week	1 month
I	500	-5°C	No growth	No growth	No growth	No growth	No growth
II	1000	-5°C	No growth	No growth	No growth	No growth	No growth
III	1500	-5°C	No growth	No growth	No growth	No growth	No growth
IV	2000	-5°C	No growth	No growth	No growth	No growth	No growth
V	nil	-5°C	28,000 per ml	26,000 per ml	27,000 per ml	23,000 per ml	19,000 per ml
II R	1000	Room Temp.	No growth	No growth	No growth	No growth	12,000 per ml.
V R	nil	Room Temp.	25,000 per ml	24,000 per ml	22,000 per ml	18,000 per ml	13,000 per ml

For testing 0.1 ml each of undiluted lymph from Nos. I, II, III, and IV and 1 ml of 1:100 dilution each of No. V, IIR and V R was used to facilitate enumeration of colonies. Most of the colonies were of the staphylococcus group. A few colonies of B. subtilis were also present. Random testing indicated that the colonies were penicillin-sensitive.

These findings were further confirmed by the following experiments.

Residual penicillin from the treated lymph was removed by alternate washing with distilled water and centrifuging four times and the sediment was tested for sterility. The plate showed numerous colonies of staphylococcus and a few B.subtilis group of organisms (almost the same colony count of untreated control lymph sample) while the unwashed lymph gave no growth whatever (Table II).

TABLE II

No.	Penicillin units per ml	Duration of contact with penicillin	colony count on lymph samples	
			Before removal of penicillin	After removal of penicillin
I	500	1 week	No growth	230 millions per ml
II	5000	1 week	No growth	238 millions per ml
C	nil (untreated lymph)		240 millions per ml	

0.1 ml of undiluted lymph was used for testing unwashed penicillin-treated lymphs and 1.0 ml of one in a million dilution was used for penicillin-removed and untreated control lymphs.

It is clear from the above experiments that the organisms are not killed but just inhibited by penicillin and once the penicillin is either removed or inactivated, the inhibited organisms show up. The residual penicillin in the vaccine lymph is only a mask.

II. EFFECT OF STREPTOMYCIN ON GLYCERINATED VACCINE LYMPH

On finding that penicillin is ineffective in reducing the bacterial contaminants of glycerine vaccine lymph, a study of the effect of streptomycin on glycerine vaccine lymph was undertaken.

In one of the experiments, streptomycin was added in concentrations varying from 500 ug to 10,000 ug per ml of glycerine vaccine lymph and preserved in the cold storage. Sterility tests on lymphs treated with streptomycin for varying periods have given encouraging results.

It was observed that there was a fall in the bacterial population proportional to the concentration of streptomycin and the duration of its contact. A staphylococcal population of 252 millions per ml before treatment, was reduced progressively with increase in concentration of streptomycin and in 5 mg concentration per ml the count came down to so low as mere 200 per ml in about 24 hours time and to 40 per ml in a week's time (Table III).

TABLE III

Sl. No. of lymph.	Conc. of Streptomycin ug per ml	Colony count per ml after	
		24 hours	1 week
I	500	10,200	7,900
II	1000	1,400	1,100
III	2000	300	100
IV	5000	240	40
V	nil	252 millions	240 millions

No appreciable difference was noted between tests done before and after removal of residual streptomycin except with reference to B.subtilis spore contaminants of the vaccine lymph (not included in the table) which manifested after removal of residual streptomycin from the streptomycin-treated lymph by alternate washing with distilled water and centrifuging six times, indicating thereby that the spores are just inhibited but not killed by the antibiotic.

In combination with 500 units of penicillin per ml as little as 500 ug of streptomycin per ml reduced the bacterial contamination to a greater extent (Table IV).

TABLE IV
Effect of combined action of Streptomycin and Penicillin

Sl. No.	Conc. of Penicillin (units per ml)	Conc. of streptomycin (ug per ml)	colony count per ml after	
			24 Hours	1 week
I	500	500	150	60
II	1000	1000	130	60
III	2000	2000	140	30
IV	nil	nil	252 millions	240 millions

Tests were done after removing the residual antibiotics to eliminate interference of penicillin in the tests. B. subtilis spores were found to be uneffected by this method also.

A study of the effects of these antibiotics under conditions optimum for their action permitting a growth phase of the bacterial flora of vaccine was taken up next.

III. EFFECTS OF STREPTOMYCIN ON VACCINE PULP AT 37° C

Vaccine material (vaccine pulp) immediately after collection from calf was diluted with 2 volumes of distilled water (no glycerol was added) and well emulsified. The emulsion was then treated with streptomycin in concentrations varying from 500 ug to 10,000 ug per ml both singly and in combination with penicillin and incubated at 37° C for 24 hours. Bacterial reduction was complete within 24 hours' incubation in 5000 ug concentrations of streptomycin singly or in combination with 1000 units of penicillin per ml. B.subtilis spores were also affected. It was found that moulds which were persisting in lower concentrations were suppressed in 10,000 ug concentration of streptomycin (Table V).

IV. EFFECT OF PENICILLIN ON VACCINE PULP AT 37° C

Penicillin in concentrations of 500 and 1000 units per ml under the same conditions brought down the staphylococcal contaminants to 250 per ml and 200 per ml respectively and the B.subtilis content to nil but allowed an abundant growth of B.coli organisms (Table V).

TABLE V

Effect of Streptomycin and Penicillin on vaccine emulsion (without addition of glycerol at 37° C for 24 hours

Sl. No. of samples	Conc. of streptomycin (ug per ml)	Conc. of penicillin (units per ml)	Bacterial count per ml.
1.	500	-	1800 staphylococci
2.	1000	-	1500 staphylococci
3.	5000	-	moulds only
4.	10000	-	nil
5.	-	500	numerous B.coli and 250 staphylococci
6.	-	1000	numerous N.coli and 200 staphylococci
7.	500	500	150 staphylococci
8.	1000	1000	120 staphylococci
9.	1000	5000	moulds only
10.	1000	10000	nil
11.	-	-	1200 millions (staphylococci), B. subtilis, B.coli and moulds.

Tests were done after removal of residual antibiotics.

V. EFFECT OF STREPTOMYCIN AND PENICILLIN IN VIVO

(a) External application of streptomycin and penicillin over the vaccinated area of the animal.

External application of streptomycin in combination with penicillin in varying concentrations over the vaccinated area of the calf twice a day for 5 days have also yielded good results. Three different concentrations of the antibiotics (100 units of penicillin and 1000 ug streptomycin per ml; 500 units of penicillin and 5000 ug streptomycin per ml and 100 units of penicillin and 10000 ug streptomycin per ml) were used on different animals. The first application was about 1 hour after vaccinating the animal and the last one about 18 hours before the scraping of the vesicles. The scraped material was emulsified in glycerine in the usual manner and

stored in the refrigerator. It was interesting to find that external application of streptomycin reduced the bacterial contamination to a very great extent, the count being not more than 200 organisms per ml of vaccine lymph in all the cases. B. subtilis spores were unaffected by this method also.

(b) Parenteral administration of the antibiotics to a vaccinated animal.

A female buffalo calf infected with vaccinia virus was administered parenterally with 40,000 ug streptomycin/kgwt. in combination with 8000 units of penicillin/kgwt. per day in 4 divided doses for 5 days. The first injection (intra-muscular) was given 6 hours after infecting the animal with vaccinia virus and the last injection about 18 hours before removal of pulp. Streptomycin in 5000 ug concentration was applied over the vaccinated area twice a day to prevent external contamination. The scraped material (vaccine pulp) was mixed with 50% glycerine and emulsified in the usual way. Sterility tests on 0.1 ml of the emulsion failed to reveal any bacteria, a colony or two of fungus being occasionally seen. The bacterial reduction by this method has been most satisfactory being much nearer to sterility than by other methods. B. subtilis spores are also affected.

VI. EFFECT OF PENICILLIN AND STREPTOMYCIN ON THE POTENCY OF VACCINE LYMPH

Vaccine lymphs treated with different concentrations of penicillin and streptomycin for varying periods, were tried for potency on rabbits periodically. The highest concentration of penicillin used was 10,000 units per ml with a contact period of about one year and of streptomycin was 10,000 ug per ml with a contact period of about 6 months in cold storage. None of the samples tested showed any loss in potency.

VII. CLINICAL TRIAL ON CHILDREN

Penicillin-treated lymphs were not tried on humans on account of their defective bacterial purity. A preliminary trial on children of streptomycin-treated lymphs (both with and without further dilution) satisfying the various standardisation tests, indicates that the vaccination 'takes' are normal and uneventful. The work is being pursued further.

DISCUSSION

Knowing that most of the bacterial contaminants of vaccine lymph are susceptible

to the action of penicillin or streptomycin or both, a detailed investigation on the effects of these antibiotics was undertaken by the author to find out if they could be used with advantage for routine purification of vaccine lymph in lieu of the existing methods.

The author has found penicillin to be ineffective in reducing the bacterial contaminants of glycerinated vaccine lymph, the number of organisms in the vaccine lymph both before and after treatment with penicillin remaining unaltered. This becomes evident when tests are done after removal of residual penicillin from the treated lymphs. It is likely that workers who have reported spectacular results about penicillin have possibly done so without taking into consideration, the interference of the residual penicillin in the tests. Chain & Duthie [5], Todd [6] and others have established that penicillin is bactericidal against susceptible organisms only during their growth phase in a nutrient medium and not on 'resting' organisms in a non-nutrient fluid. The bacteria in glycerine vaccine lymph are in a 'resting' phase in a non-nutrient medium which explains the failure of penicillin in bringing down the bacterial flora in vaccine lymph.

On the other hand Streptomycin (in 5 mg concentration per ml) effectively brings down the bacterial contamination in glycerine vaccine lymph. But the B.subtilis organisms which occur as spores in the glycerine lymph are unaffected. Strauss Garrod[8] have clearly demonstrated that streptomycin is definitely bactericidal both in nutrient and non-nutrient fluids, though in higher concentrations in the latter. Streptomycin is also more versatile in being active both against gram-positive and gram-negative organisms. Experiments with streptomycin have further indicated that acting synergistically with penicillin as little as 500 units of penicillin per ml produce equally effective bacterial reduction of vaccine lymph. Chain & Duthie [5], Himmelweit [9] and Pulaski [10] et al report that certain antibiotics in combination with sulphonamides, bacteriophage or antibiotics show such a synergistic action.

The study of the effects of these antibiotics under conditions optimum for their action, permitting a growth phase of the bacterial flora of vaccine lymph, have yielded interesting results. 5000 ug of streptomycin either singly or in combination with 1000 units of penicillin per ml added to vaccine pulp immediately after its collection from the calf (but without addition of glycerol) and incubated for 24 hours reduces entirely all the bacterial flora. B.subtilis spores are also affected. It is just likely that the B.subtilis spores develop into vegetative forms under such favourable

conditions, only to become vulnerable to the action of streptomycin and penicillin. A few moulds persisting in lower concentrations, are found to be suppressed in 10,000 ug. concentrations of streptomycin.

Penicillin by itself under the growth phase of the organisms in vaccine lymphs brings down the susceptible contaminants but permits on abundant growth of B.coli organisms. Thus penicillin fails to be of any value even under this condition, due to diverse nature of bacterial flora of vaccine lymph.

Mere external application of streptomycin in combination with penicillin over the vaccinated area of an animal reduces the bacterial contamination to a substantial extent.

Parenteral administration of streptomycin and penicillin combined in suitable concentrations bring the bacterial reduction nearer to sterility than by other methods including the B.subtilis spores.

Thus streptomycin when employed under all the above conditions brings down the bacterial contamination of vaccine lymph to a satisfactory extent and in some cases nearer to sterility. While the Therapeutic Substances Act permits up to 5,000 organisms per ml of lymph, streptomycin-treated lymphs have hardly 200 organisms or less per ml and in some cases no bacteria at all.

The potency of vaccine lymph is unaffected both by penicillin and streptomycin under these conditions. While the minimal requirement for a consistent successful vaccination 'take' on child in tropics, is a 10^{-4} or 10^{-5} positive potency (continuous vaccinal vesiculations on rabbit), the streptomycin-treated lymphs have usually a 10^{-8} positive potency or more. This will permit the usage of highly diluted vaccines, thereby effecting great savings in production cost. Further, these findings promise application in the production of dry vaccine also.

SUMMARY

The effects of penicillin and streptomycin on vaccine lymph (calf lymph) under different conditions are described.

Penicillin is ineffective in reducing the bacterial flora of vaccine lymph under these conditions; this becomes evident only when tests are conducted after removal of residual penicillin from the penicillin-treated lymphs.

The author is unable to confirm the spectacular reports of some workers who seem to have based their observation ignoring the interference of residual penicillin in the test.

Streptomycin under the same conditions successfully brings down the bacterial flora of the vaccine lymph to the required extent.

Both streptomycin and penicillin do not affect the potency of vaccine lymph.

The application of these findings in the preparation of purer calf lymph at a substantially lesser cost than at present is discussed.

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Lilly's crystalline penicillin and Merck's streptomycin (calcium chloride complex) were used all through except for experiments on parenteral administration for which Pfizer's Dihydrostreptomycin sulphate was employed.

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