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Serial subcultivation of Czechoslovakian and Japanese BCG strains

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SUMMARY

Changes in the Danish BCG strain under certain regimens of subculture have been shown in preceding studies to be associated with selection of a minority population. Three Czechoslovakian BCG strains, all originally derived from the Danish strain but thereafter and in distinction from it maintained on potato media, have now been investigated. Changes in the immunizing potency of two of these strains have been attributed by other workers to employment of the richer potato media in place of Sauton medium as used for maintenance of the parent Danish strain. However, results from the present study suggest rather that selection of a pre-existing minority genotype or of a new mutant occurred. This proposal is supported by the finding that the third strain has maintained characteristics similar to those of the Danish parent despite many previous transfers on potato media.

Another BCG strain investigated was the Japanese which, like the three Czechoslovakian strains, had been previously maintained on potato media. This strain has been shown in the present study to resemble the Danish strain in supporting a minority population yielding non-spreading colonies. Czechoslovakian vaccine prepared with seed culture supplied from Tokyo has retained characteristics similar to those of the Japanese parent. Although a majority population yielding spreading colonies appears so far to have been retained in both centres, it is considered that selection of the minority could still occur in the course of routine transfer.

INTRODUCTION

Since 1919, when Calmette and Guérin considered that attenuation of their strain was complete, culture samples of BCG have been supplied by the Pasteur Institute to centres in many different parts of the world, each of which set up its own line of transfer for the production of BCG vaccine. Several of these centres have in turn supplied seed culture to other countries; for example, the State Serum Institute, Copenhagen, has supplied a number of other laboratories including those in Great Britain and Czechoslovakia, and the Japan BCG Laboratory, Tokyo, has also supplied seed culture to the latter. It is well known that daughter strains of BCG, maintained under a variety of conditions, have come to differ considerably in some of their characteristics; thus in laboratory studies the

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British and Czechoslovakian strains have both shown a lower immunizing potency than their parent Danish strain (Ladefoged, Bunch-Christensen & Guld, 1970; Průchová *et al.* 1976). Some light has been thrown on the mechanism of change in two recent studies of the sequence of events when BCG strain 1331 (Copenhagen) was experimentally re-introduced into the British production procedure (Osborn, 1976, 1979). During small-scale reconstructions in this laboratory of the culture sequence employed for production, it was observed that strain 1331 (Copenhagen) could change to yield a majority of the non-spreading colonies characteristic of the British strain 1077 (Glaxo) in place of the spreading colonies normally cultivated from the Danish strain. In the course of the production of BCG vaccine in Britain the seed culture is first subcultured several times on the surface of Löwenstein-Jensen medium, followed by sequential deep transfers in two different liquid media. It was found that the small minority population yielding non-spreading colonies carried by the Danish strain could be readily selected during transfer on Löwenstein-Jensen medium and that this effect was further accentuated by subsequent transfer in deep culture in one of the liquid media. The parent Danish strain itself had been maintained in Copenhagen for a period of 17 years by transfer as a surface pellicle on liquid Sauton medium and it is noteworthy that, notwithstanding over 600 such transfers, the minority population had not been selected. In fact, studies in this laboratory have indicated that subcultivation of the Danish strain as a surface pellicle on Sauton medium has a powerful effect in reducing a minority population of moderate proportions virtually to vanishing point (Osborn, 1979).

Most centres throughout the world have employed potato media for the maintenance of their BCG strains rather than the media used in Great Britain and Denmark, yet in some of these laboratories, too, changes have occurred. For example, the Czechoslovakian BCG strain employed for the production of vaccine has been maintained in the traditional way on potato Sauton medium and, as stated above, was found by workers in Prague to show a lower immunizing potency than that of the parent Danish strain. On the other hand, these workers have found that another line, derived at a later date from the Danish strain but also maintained on potato Sauton medium, shows an immunizing potency similar to that of the parent Danish strain. A smaller number of centres has maintained BCG lines on potato bile medium and one of these is the BCG laboratory in Tokyo. As a further step towards understanding changes in BCG it was clearly desirable to examine the behaviour on subculture of lines that had previously been maintained on potato media, and hence strains from Prague and Tokyo that had been transferred in this way are the subject of the present report.

MATERIALS AND METHODS

BCG vaccines

(A) Danish freeze-dried BCG vaccine prepared by the State Serum Institute, Copenhagen, Denmark from strain 1331 (Copenhagen).

(B) Czechoslovakian freeze-dried BCG vaccine prepared by the Institute of Hygiene and Epidemiology, Prague, Czechoslovakia.

(i.*a*) From a line seeded in 1947 with culture sent to Prague from the 725th transfer of the Danish BCG line. After seeding onto potato Sauton medium the line was maintained by transfers from the surface of potato in one container to the surface of potato in the next, until about 1974, when the strain was freeze-dried. The Sauton medium employed for the maintenance of this strain contained asparagine, according to the original formula. The strain from this line is not now employed for the production of vaccine and is known in Prague as *strain 725SAS*.

(i.*b*) From a line seeded in 1950 with culture from strain 725SAS onto potato Sauton medium in which the asparagine of the original Sauton formula had been replaced by enzymic hydrolysate of casein. The line was maintained in a similar way to that of strain 725SAS until 1967 when it was freeze-dried. It is used for vaccine production and the strain is known in Prague as *strain 725SH*.

(ii) From a line seeded in 1950 with culture sent to Prague from the 901st transfer of the Danish BCG line. After seeding onto potato Sauton medium (prepared with enzymic hydrolysate of casein) the strain was maintained by serial transfers on this medium until it was freeze-dried a few years ago. This strain, which is not now employed for vaccine production, is known in Prague as *strain 901SH*.

(iii) From a freeze-dried seed lot, prepared in Prague in 1969 using as seed suspension taken from an ampoule of the World Health Organization's first reference preparation of BCG vaccine, 1965, which in its turn had been prepared in Tokyo from seed of Japanese strain 172. (World Health Organization, 1972).

(C) Japanese freeze-dried BCG vaccine prepared by the Japan BCG Laboratory, Tokyo, Japan, from strain 172 (Tokyo). The preparation used in this study was the World Health Organization's first reference preparation of BCG vaccine, 1965, also employed as seed in the preparation of one of the Czechoslovakian BCG vaccines (see above). Sub-lots C and E of this vaccine were examined.

Examination of colony morphology

Media

The composition of the modified Dubos-type agar medium enriched with stored human blood used for the study of colony morphology has been described elsewhere (Osborn, 1976). Since that report it has become clear that Sauton medium, employed for reconstitution and dilution of freeze-dried BCG vaccines, has an important influence on colony morphology and that the key constituent in this respect is glycerol.

Procedure

Freeze-dried BCG vaccine in ampoules was reconstituted with Sauton medium and suitably diluted suspensions in this medium were seeded by means of a calibrated dropper onto the surface of the blood-enriched agar medium contained in plastic Petri dishes, Cultures taken from serial transfers were also suitably diluted and seeded onto the surface of this medium. When the drops had dried the Petri dishes were sealed in plastic bags and were then incubated at 37°C in the inverted position for 28 days.

At the end of the incubation period and at the optimum dilution (giving approximately 10 colonies per drop) the morphology of the mature colonies cultured on the surface of the medium was studied with the aid of a binocular microscope.

Serial subculture

Reconstituted BCG vaccine from ampoules provided seed for the initiation of serial transfers with the following media:

Löwenstein-Jensen slopes in universal containers

After seeding each container was incubated at 37°C for approximately 28 days. A portion of culture was then removed and transferred to the surface of the medium in a further container; another portion was removed and transferred to a small bottle holding glass beads and Sauton medium in which it was shaken to disperse the culture. The dispersed culture was then further diluted and plated out for the examination of colony morphology.

Sauton medium in 100 ml conical flasks

After seed had been inoculated on the surface each flask was incubated at 37°C for approximately 21 days. Following incubation the surface pellicle was harvested and a portion was seeded on the surface of Sauton medium in a further flask; the remainder was dispersed by shaking the medium and was then further diluted and plated out for the examination of colony morphology. Culture of the Czechoslovakian BCG lines was also made on Sauton medium in which the usual asparagine constituent had been replaced by 0·75% enzymic digest of casein. (Kindly supplied by the Prague Laboratory.)

Dubos and production media in 100 ml flat bottles

Reconstituted BCG vaccine was introduced into serial deep subculture in Dubos medium which contained the surfactant Tween 80 in a concentration of 0·05%, and also in a medium employed in the preparation of British BCG vaccine, known as production medium, which contained the surfactant Tyloxapol (Triton WR 1339) in a concentration of 0·025% (Ungar *et al.* 1962). After incubation for approximately 10 days at 37°C the culture was dispersed by shaking; a portion of the dispersed culture was then transferred to a further container of Dubos or production medium and a portion of the remainder was appropriately diluted and plated out for the examination of colony morphology.

RESULTS

The much improved separation of colony forms that can be obtained by cultivating BCG vaccines on the modified Dubos-type agar medium employed in this laboratory was described and illustrated in the publication already cited (Osborn, 1976). In the study which was the subject of that report the culture technique was employed to distinguish between the colonies normally cultured from BCG strain

1331 (Copenhagen) and those from BCG strain 1077 (Glaxo). It has subsequently been found to be of value in distinguishing between the majority population of spreading-type colonies culturable from strain 172 (Tokyo) and a minority population of non-spreading colonies. A clear distinction can also be made between colonies cultured from strain 725 (Prague) and those from the parent Danish strain. The descriptions 'spreading' and 'non-spreading' as used by Pierce & Dubos (1956) are retained to assist in the identification of the various colony forms. The characteristic appearances are much more evident under a binocular microscope than in photographs.

Examination of cultures made directly from vaccine preparations

The colonies cultured from vaccine reconstituted from ampoules, suitably diluted and then inoculated onto the surface of our modified Dubos-type agar medium, were examined under a binocular microscope at the end of the incubation period.

(*a*) *Danish vaccine strain* 1331 (*Copenhagen*). Spreading colonies constituted > 99 % of the population (Plate 1A).

(*b*) *Czechoslovakian vaccine strain* 725*SAS and strain* 725*SH*. The spreading colonies cultured from both strains were indistinguishable from each other but were smaller than those isolated from the parent Danish strain (Plate 1B). They constituted > 99 % of the population and had a characteristic waxy appearance when viewed under a binocular microscope.

(*c*) *Czechoslovakian vaccine strain* 901*SH*. Spreading colonies constituted > 99 % of the population, and were indistinguishable from those of the parent Danish strain (Plate 1A).

(*d*) *Czechoslovakian vaccine prepared from Japanese strain* 172. Spreading colonies constituted > 99 % of the population, and were indistinguishable from those cultured from the parent Japanese strain (Plate 1C). These colonies were readily distinguished from those of the other Czechoslovakian strains and from those of the Danish strain on account of their size and surface texture.

(*e*) *Japanese vaccine strain* 172 (*Tokyo*): the WHO first reference preparation of BCG vaccine, 1965.

Sub-lot C: spreading colonies constituted > 99 % of the population and were indistinguishable from those cultured from the Czechoslovakian vaccine prepared from strain 172 (Tokyo) (Plate 1C).

Sub-lot E: spreading colonies indistinguishable from those cultured from sub-lot C constituted only 90 % of the population. The remaining 10 % consisted of non-spreading colonies indistinguishable from those isolated from the Czechoslovakian vaccine prepared from strain 172 (Tokyo) after transfers in Dubos medium (Plate 1D).

Examination of cultures made during serial transfers

(*a*) *Danish vaccine strain* 1331 (*Copenhagen*). The effects of serial subcultures made with this strain on Löwenstein-Jensen, Sauton, Dubos and production media have already been described (Osborn, 1976, 1979), and were not further

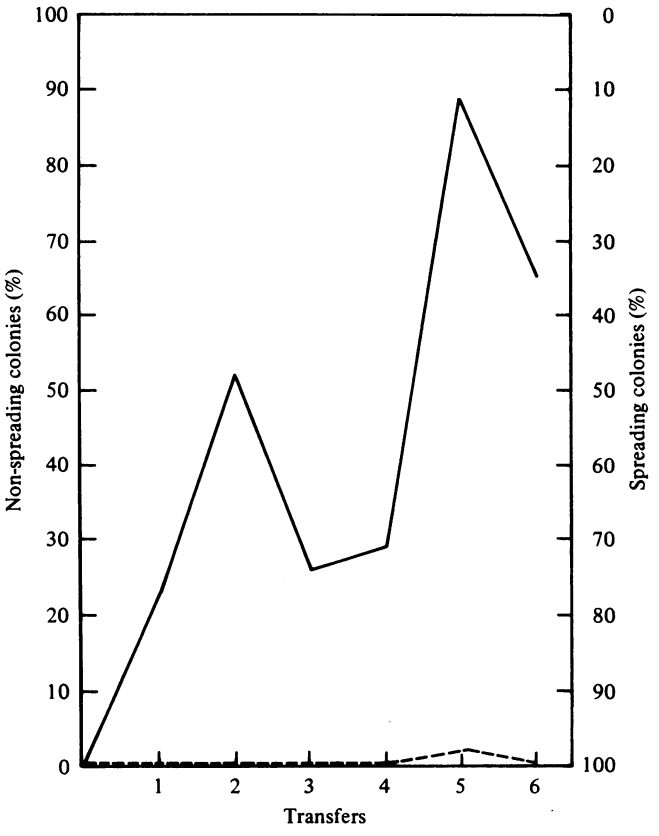

Fig. 1. Relative proportions of spreading and non-spreading colonies cultured from serial transfers seeded with Czechoslovakian BCG vaccine strain 901SH in Dubos medium (——) and in production medium (– – –).

examined in the present study. It was found in these earlier investigations that selection of a minority population could readily take place in the course of transfers on the surface of Löwenstein-Jensen medium and such selection, when it occurred, appeared to be related to the non-homogeneous nature of the culture. With deep subculture, on the other hand, transfer in Dubos medium positively favoured selection of an original minority yielding non-spreading colonies, whilst subsequent transfer in production medium promoted a return to a majority of spreading forms. However, during serial transfer as a surface pellicle on Sauton medium virtually 100 % spreading-type colonies was regularly obtained.

(*b*) *Czechoslovakian vaccine strains* 725*SAS and* 725*SH*. The small spreading colonies initially cultured, and indistinguishable between the two strains, remained unchanged during serial subculture on Löwenstein-Jensen medium and in Dubos and production media. There was also no change during transfers on Sauton medium, whether the latter contained asparagine according to the usual formula, or enzymic digest of casein in its place.

(*c*) *Czechoslovakian vaccine strain* 901*SH*. The effects of transfer on Löwenstein-Jensen medium were not studied but in the course of deep subculture in Dubos

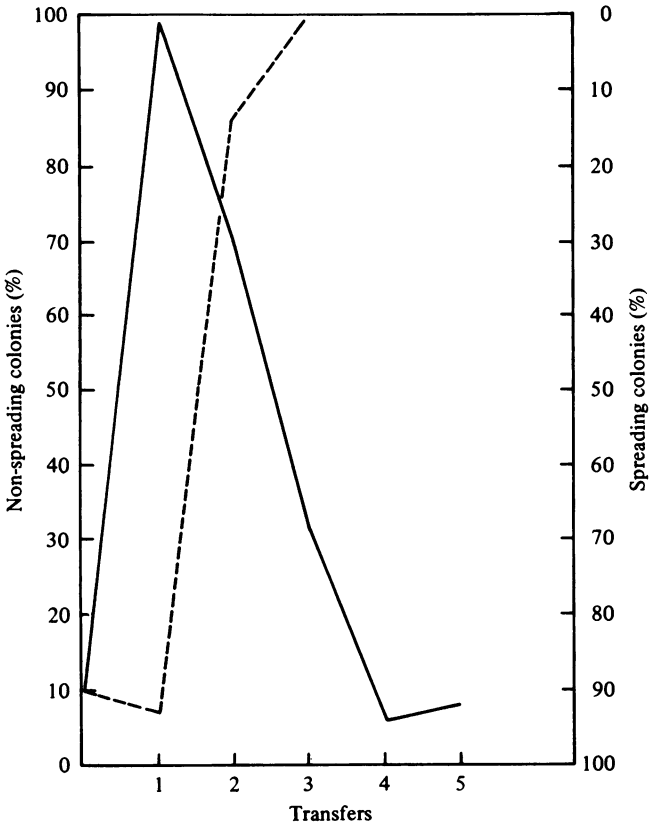

Fig. 2. Relative proportions of spreading and non-spreading colonies cultured from serial transfers on the surface of Löwenstein–Jensen medium seeded with Japanese strain 172 (Tokyo), sub-lot E of the WHO first reference preparation of BCG vaccine, 1965. ——, line 1; – – –, line 2.

medium there was progressive selection of the orginally minute population of non-spreading colonies, whilst the overwhelming majority of spreading colonies as cultured from the seed vaccine was maintained throughout during deep transfer in production medium (Fig. 1). The > 99 % majority of spreading colonies was maintained during serial transfer on the surface of Sauton medium. The behaviour of strain 901*SH* during serial subculture in Dubos and production media and on the surface of Sauton medium thus entirely matched that seen during previous extensive studies with the parent Danish strain (Osborn, 1976, 1979).

(*d*) *Czechoslovakian vaccine prepared from Japanese strain* 172. The effects of subculture on Löwenstein-Jensen medium were not studied, but the results of subculture in Dubos and production media and on Sauton medium matched those recorded for the parent Japanese strain (see below).

(*e*) *Japanese vaccine strain* 172 (*Tokyo*), the WHO first reference preparation of BCG vaccine, 1965. The effects of serial subculture on all of the media employed were similar for sub-lot C, which showed an initial > 99 % of spreading colonies, and for sub-lot E, which showed an initial 90 % of spreading colonies, with 10 % of non-spreading forms. Results of subculture from sub-lot E on Löwenstein-

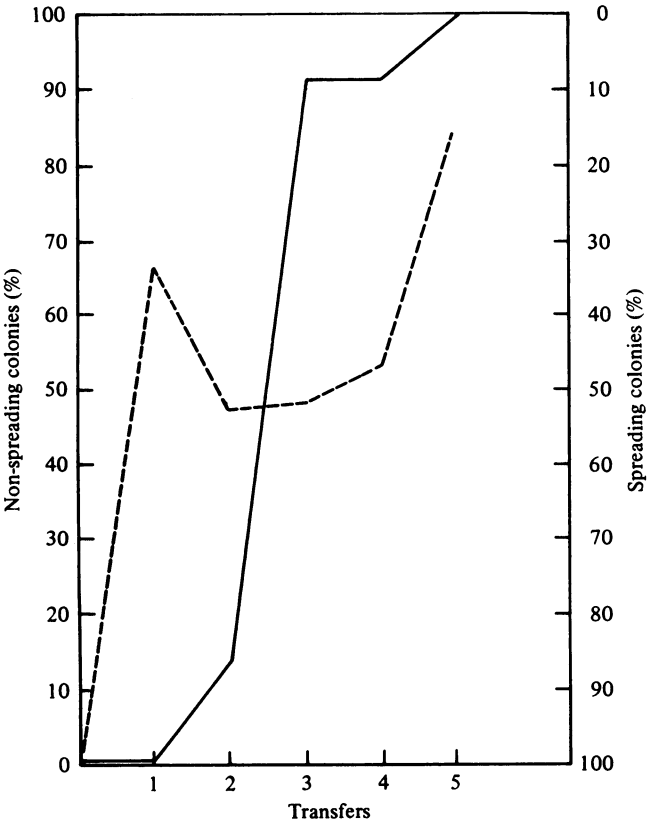

Fig. 3. Relative proportions of spreading and non-spreading colonies cultured from serial transfers seeded with Japanese strain 172 (Tokyo), sub-lot C of the WHO first reference preparation of BCG vaccine in Dubos medium (——) and in production medium (– – –).

Jensen medium employing two lines of transfer, and representative of both sub-lots, are shown in Fig. 2. As was previously found with strain 1331 (Copenhagen) when cultivated on this medium (Osborn, 1979), rapid changes can occur in the balance between spreading and non-spreading colonies, but they appear to be fortuitous, and to be related to the lack of homogeneity in the structure of the surface culture. Results from deep subculture in Dubos and production media representative of both sub-lots are shown for sub-lot C in Fig. 3. Again, as was previously found in the case of strain 1331 (Copenhagen), subculture in Dubos medium favoured selection of the originally small minority population yielding non-spreading colonies. In contrast, however, from the findings with the Danish strain, transfer in production medium also favoured selection of these non-spreading forms. The results for both sub-lots of the vaccine of serial subculture as a surface pellicle on Sauton medium matched those previously obtained with strain 1331 (Copenhagen) in rapidly reducing a minority population yielding non-spreading colonies almost to vanishing-point and thereafter maintaining a yield of virtually 100 % of the spreading form.

DISCUSSION

If changes in strains of BCG are to be minimized in the future, more understanding is needed of the ways such changes occur, and how they may relate to factors common to different regimens of maintenance. Previous studies have demonstrated that the Danish BCG strain carries a very small minority population that on culture yields non-spreading colonies, but that serial transfer as a surface pellicle on Sauton medium, as practised in Copenhagen, favours maintenance of the majority population yielding spreading colonies (Osborn, 1979). However, when the Danish strain is introduced as seed into another production procedure involving transfers on the surface of Löwenstein-Jensen medium followed by deep subcultures in two different liquid media, selection of the minority population readily occurs (Osborn, 1976).

Most BCG production centres do not use the latter technique and their procedures differ also from that employed in Copenhagen in utilizing the traditional potato-Sauton or potato-bile media in addition to Sauton medium. Thus the Pasteur Institute itself for many years employed transfers on potato-Sauton medium, with occasional transfers on potato-bile medium in the maintenance of its BCG strain (M. Gheorghiu, personal communication) and the earlier studies in this laboratory indicated that the Pasteur strain does not contain a readily detectable minority population and apparently remains stable during subculture (Osborn, 1976, 1979). However, the Czechoslovakian BCG strain, maintained in a similar manner on potato-Sauton medium but without the occasional transfers on potato-bile medium employed at the Pasteur Institute (J. Galliová, personal communication), has been found by the workers in Prague to differ appreciably from its Danish parent (Průchová *et al.* 1976).

The Czechoslovakian workers maintained three lines that had originally been initiated with seed from the Danish BCG strain, and the strains derived from them are known in Prague as 725SAS, 725SH and 901SH (see Materials and Methods). In the present study, culture from strains 725SAS and 725SH on the modified Dubos-type agar medium employed in this laboratory yielded small spreading-type colonies identical in appearance, which did not change during serial transfers with four different media. Furthermore, the appearance of these colonies could be readily distinguished from that of the larger spreading colonies of strain 901SH, which themselves appeared identical with those isolated from the parent Danish BCG strain. In this connexion it is of interest that a minority population yielding non-spreading colonies was selected from strain 901SH during deep subculture in Dubos medium, whilst the overwhelming majority of spreading forms culturable from the seed vaccine was maintained throughout during deep transfer in production medium or as a pellicle on the surface of Sauton medium; in these respects also strain 901SH is similar to the parent Danish strain (Osborn, 1976, 1979).

The Czechoslovakian workers found the immunizing potency of strain 901SH to be similar to that of the parent Danish strain whilst that of strains 725SAS and 725SH was lower; they also found the microscopic appearance of the bacilli of the latter two strains to be similar and different from that of the bacilli of strain

901SH which resembled that of the parent Danish strain (Průchová *et al.* 1976). The workers in Prague attributed changes in strains 725SAS and 725SH to cultivation on richer media than that employed for maintenance of the parent Danish strain. Thus Sauton's original medium, employed for cultivation of the latter strain contains only one amino acid, asparagine, whilst the culture medium employed for the maintenance of strains 725SAS and 725SH was considerably enriched by the biochemical components of potato, and, furthermore, the modified Sauton medium with the potato slopes employed for the maintenance of strain 725SH contained casein hydrolysate, which has a richer content of various amino acids.

The results obtained in the present study, in the light of those from earlier studies made in this laboratory, suggest that the similarities of strains 725SAS and 725SH may be attributable to selection of a genotype, which was either already in existence as a minority carried in the parent Danish strain maintained in Copenhagen, or arose as a mutant after subcultures were started in Prague, *before* 1950, when separate maintenance of the two 725 lines commenced. The interesting findings of the workers in Prague, on the other hand, might be attributable to changes of a phenotypic character and this would be supported by their own observation that the changes they describe appeared to be unstable. Furthermore, Galliová (1971) has very clearly demonstrated that the parent Danish BCG strain, maintained in Copenhagen on Sauton medium prepared with asparagine, shows a constitution of lipids and proteins similar to that of strain 725SAS also maintained on this medium, despite their distinct differences in immunogenic potency. Likewise, strain 901SH maintained on Sauton medium prepared with enzymic digest of casein shows a constitution of lipids and proteins similar to that of strain 725SH, maintained in the same way, again despite differences in immunogenic potency. Indeed, the difference of immunogenic potency between the latter two strains, and the similarity in this respect of strain 901SH to the parent Danish strain clearly suggests that maintenance on the richer potato medium *per se* is not responsible for diminution in such potency.

The Japanese BCG strain was established from seed supplied directly from the Pasteur Institute strain in 1925 and was thereafter maintained by serial subculture on bile-potato medium (T. Sawada, personal communication). A freeze-dried lot of BCG vaccine, consisting of sub-lots A–E, was prepared in Tokyo in 1960 from the 172nd transfer on bile potato medium, and the strain is known as strain 172 (Tokyo). This lot is of particular interest as it was adopted as the first reference preparation of BCG vaccine, 1965 (World Health Organization, 1972), and samples from sub-lots C and E were therefore included in the present study. As was found in the earlier studies in this laboratory with the Danish BCG strain, the Japanese strain also carries a minority population that on culture yields non-spreading colonies and the appearance of these colonies was very similar, but not identical, in the two strains. Again, as was previously found with the Danish strain, an apparently fortuitous selection of the minority population could occur during subculture on the surface of Löwenstein-Jensen medium, whilst deep transfer in Dubos medium definitely favoured such selection. However, in distinction from

the Danish strain, with which deep subculture in production medium favoured the majority population yielding spreading colonies, with the Japanese strain, transfer in this medium was similar to transfer in Dubos medium in favouring the population yielding non-spreading forms. The powerful effect favouring maintenance of the original majority population yielding spreading colonies seen previously with the Danish strain during subculture as a pellicle on the surface of Sauton medium was again evident in the case of the Japanese strain. That the process of freeze-drying itself can influence the balance between the original majority and minority populations is indicated by the fact that vaccine from sub-lot C on culture yielded $> 99\%$ of spreading colonies, with a minute population of non-spreading forms, whilst the latter constituted 10 % of those isolated from sub-lot E. This was despite the fact that, although they were freeze-dried in separate machines, both sub-lots had been prepared from the same final bulk of vaccine material (T. Sawada, personal communication). Strain 172 (Tokyo) in the hands of the Czechoslovakian workers was maintained by subculture on potato-Sauton medium as a preliminary to preparing a freeze-dried seed lot. In the present study the characteristics of the vaccine prepared in Prague from this strain entirely matched those of sub-lot C of the vaccine prepared in Tokyo in yielding on culture $> 99\%$ of spreading colonies. Maintenance of freeze-dried seed lots in Tokyo and Prague can be expected to reduce the opportunities for changes in the strain; nevertheless, as has been previously demonstrated with the Danish strain, a virtually complete change can occur within a single production cycle, and indeed this recently happened when the Japanese strain was experimentally introduced into the production procedure of another manufacturer (manuscript in preparation).

The results of the present study with the Danish, Czechoslovakian and Japanese vaccines are entirely consistent with the findings from earlier studies made in this laboratory, which demonstrated that selection of a minority population is an important factor in promoting changes in a BCG strain. Such selection rather than maintenance on culture media of differing constitution appears more likely to be responsible for changes in the immunizing potency of BCG strains. Ways in which such changes might be prevented will be discussed in a forthcoming publication.

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EXPLANATION OF PLATE

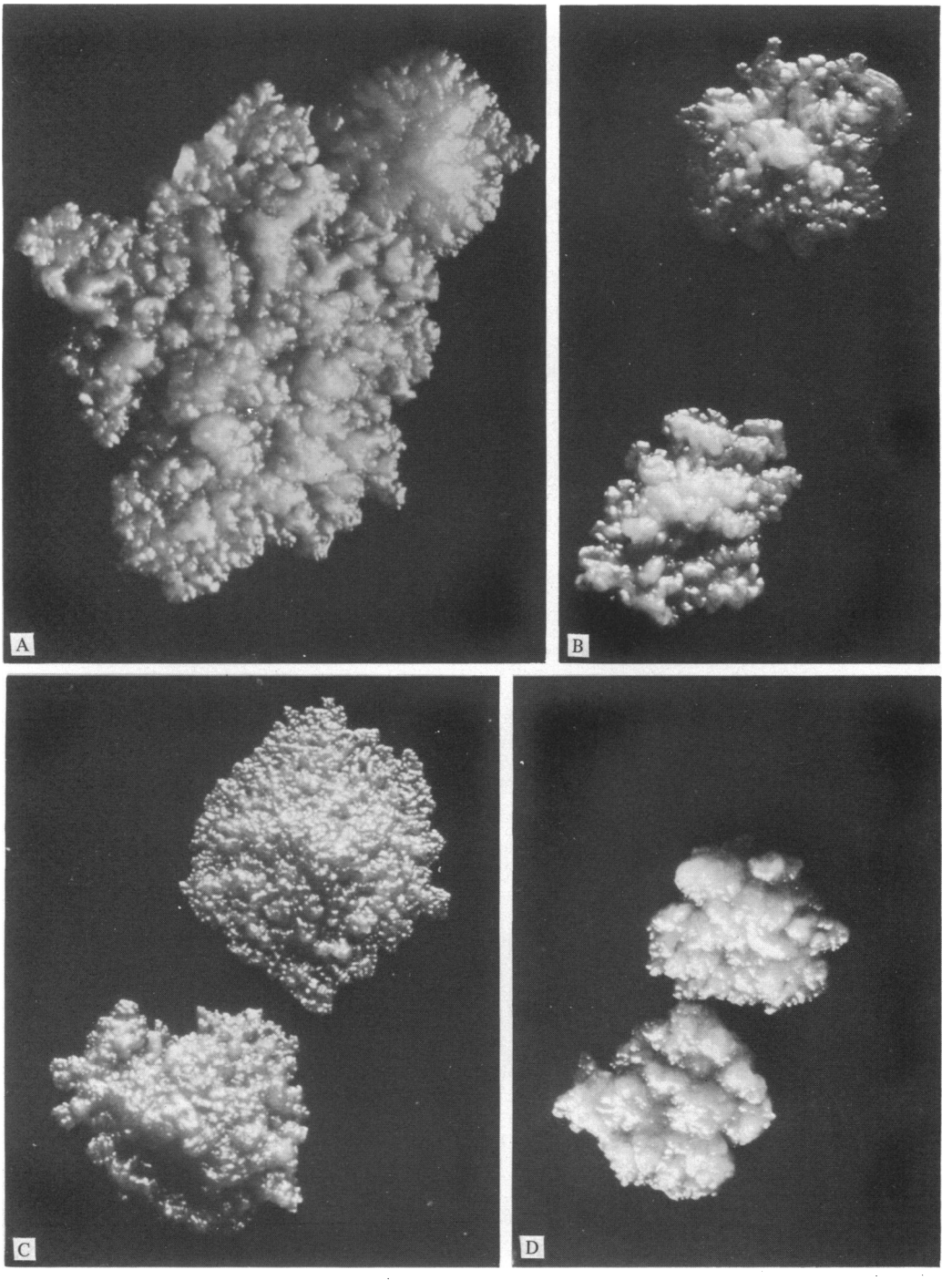
PLATE 1

(A) A spreading colony in contact with a non-spreading colony (top) cultured from Czechoslovakian strain 901SH after several transfers in Dubos medium. The spreading colonies appeared indistinguishable from those normally isolated from the parent Danish strain, and the non-spreading forms were indistinguishable from those that came to predominate in the parent strain after similar subculture in Dubos medium. × 15.

(B) Spreading colonies cultured from Czechoslovakian strain 725SAS. The colonies are similar in appearance to those isolated from Czechoslovakian strain 725SH and smaller than those cultured from the parent Danish strain. × 15.

(C) Spreading colonies cultured from Czechoslovakian vaccine prepared from strain 172 (Tokyo). These are similar in appearance to those isolated from the parent Japanese strain. × 15.

(D) Non-spreading colonies cultured from Czechoslovakian vaccine prepared from strain 172 (Tokyo) after transfers in Dubos medium. These appear indistinguishable from colonies that can be similarly cultured from the parent Japanese strain after transfers in that medium. × 15.



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