ISOLATION OF *BACILLUS LICHENIFORMIS* VAR. *ENDOPARASITICUS* FROM THE BLOOD OF RHEUMATOID ARTHRITIS PATIENTS AND NORMAL SUBJECTS

ROBERTA BARTLETT AND K. A. BISSET

Department of Bacteriology, University of Birmingham, Birmingham B15 2TJ

SUMMARY. Sixty-five samples of blood from patients with rheumatoid arthritis and 94 from control subjects were examined by multiple culture and monthly subculture for periods of up to 33 months to detect the reversion stages of *Bacillus licheniformis* var. *endoparasiticus* (BLE), which exists as L-forms in blood.

Isolates of BLE were obtained more often from the blood of control subjects than from rheumatoid patients during the first 6 months of incubation, when there was clustering of positive cultures within samples. Thereafter, the isolation rate was similar for the two groups and positive cultures were distributed randomly between samples. Isolation of diphtheroid intermediate reversion stages (phases A and B) occurred mainly during the first year of incubation, but isolations of the fully reverted sporing bacillus (phase C) increased in frequency with incubation time, particularly from cultures with a high rate of desiccation during prolonged incubation. The proportion of different phases of BLE amongst the isolates and the distribution of the phases with incubation time were similar for the rheumatoid-arthritis patients and the normal subjects.

INTRODUCTION

Bacillus licheniformis var. *endoparasiticus* (Benedek) occurs as an L-form associated with the erythrocytes of a large proportion of clinically normal persons. It is often exceedingly slow to revert and is not usually detected without prolonged incubation. When it is detected it is likely to be disregarded as a contaminant (Bisset and Bartlett, 1978; Bartlett and Bisset, 1979; Bisset, Tallack and Bartlett, 1979).

It has been postulated that chronic infection with bacterial L-forms or mycoplasma may contribute to the failure of normal immunological recognition of tissues and the production of autoantibodies in autoimmune diseases, including rheumatoid arthritis (Pease, 1965, 1967; Brown, Clark and Bailey, 1975). Diphtheroids (Stewart, Alexander and Duthie, 1969; Duthie, Stewart and McBride, 1976) and *Mycoplasma* (Bartholomew, 1965; Jansson *et al.*, 1971) have been isolated more often from synovial membranes and fluids of

rheumatoid arthritis patients than from those of patients with non-rheumatoid joint conditions. Bartholomew and Nelson (1972) have reported a higher isolation rate of the anaerobic or microaerophilic "*Corynebacterium acnes*" from the blood of rheumatoid patients than from non-rheumatoids.

Previous work in this laboratory (Pease, 1974; Bisset and Bartlett, 1978) has produced evidence that the organisms resembling *Mycoplasma* and those described as diphtheroid bacteria, isolated from the blood of rheumatoid patients and from normal persons (Benedek, 1955; Pease, 1969, 1970; Duthie *et al.*, 1976; Markham and Myers, 1976; Tedeschi *et al.*, 1978), are L-forms and reversion stages of *B. licheniformis* var. *endoparasiticus*, hereafter referred to as BLE.

MATERIALS AND METHODS

Blood samples

Collection of samples. A total of 164 blood samples were examined, 65 were from rheumatoid arthritis patients, five from patients with other arthritic conditions, and 94 from controls.

Fifty-five samples of blood from healthy donors were taken by courtesy of the West Midland Regional Blood Transfusion Service; 10-ml samples were collected in 1-ml sterile acid-citrate-dextrose anticoagulant at the end of the donation from the tube that remained connected to the donor's vein, by the method and with the precautions previously described (Bisset and Bartlett, 1978).

Twenty-one samples of blood from laboratory staff were taken by Dr M. Synnott of the Department of Medical Microbiology, University of Birmingham. The donor's skin was disinfected with 70% isopropyl alcohol (Sterets Injection Swab, Prebbles Medical Ltd, Merseyside); 5 ml of blood was taken into disposable hypodermic syringes and transferred to sterile screw-capped bottles containing 0.25 ml of 0.4% (w/v) aqueous heparin.

The same technique was used for the collection of two samples of blood from laboratory staff by the Haematology Department, Queen Elizabeth Hospital, Birmingham, and 15 samples of blood taken by the Haematology Department, General Hospital, Birmingham from carefully selected elderly outpatients suffering from non-infective, non-autoimmune conditions unrelated to rheumatism or arthritis, who were regarded as being clinically normal for the purpose of this experiment and were included in the study to provide an age distribution in the control group similar to that of the rheumatoid-arthritis patients.

Seventy-one samples of blood from patients attending an arthritis clinic were supplied by Dr M. Farr of the Queen Elizabeth Hospital, Birmingham. Samples were taken by syringe and transferred to containers with either acid-citrate-dextrose or heparin anticoagulant, as described above. Sixty-five samples were from patients with rheumatoid arthritis, three from patients with osteoarthritis, two from patients with Crohn's disease, and one from a patient with a physical injury, who was regarded as clinically normal for the purpose of this experiment.

Anticoagulants. All bottles containing anticoagulant were provided by and prepared in this laboratory, and regularly tested for sterility. Bottles containing acid-citrate-dextrose were autoclaved at 121°C for 20 min and those containing heparin at 118°C for 10 min.

Culture methods

Inoculation. Primary cultures were made either from untreated blood, or from red cells harvested by low-speed centrifugation, washed three times with sterile saline, and resuspended in the same volume of sterile saline. One ml of whole blood or washed cells was added to 10 ml of the L-form medium described by Pease (1970), which had been sterilised by autoclaving at 121°C for 20 min. The number of cultures from any one sample ranged from one to nine; when more

than one culture was made from a single sample, they were labelled sequentially at the time of inoculation.

Incubation. Cultures were incubated at 37°C, with the bottle caps firmly screwed down to minimise evaporation, for periods of up to 33 months; incubation times varied widely because samples were obtained at different times during the course of the experiment.

Subculture. At monthly intervals, cultures were examined visually and subcultured on to Trypticase Soy Agar (Difco). Plates were incubated aerobically inside plastic bags at 37°C for 2 weeks. All cultures yielding visible growth on subculture were subcultured again and the isolates identified. The time at which cultures produced isolates, or dried up, was noted.

Throughout incubation, some cultures were incubated and subcultured by one experimenter (R.B.) and others by a succession of other workers in different rooms and using different incubators.

Identification of isolates

Isolates were identified by the following physiological tests: motility; growth on MacConkey agar; Voges-Proskauer reaction; production of oxidase, catalase, urease and indole; hydrolysis of starch, casein and gelatin; utilisation of citrate; reduction of nitrate; Hugh and Leifson's oxidation-fermentation test; anaerobic growth in glucose broth; tolerance of 10% NaCl; fermentation of glucose, sucrose, lactose, maltose, mannitol, dulcitol, arabinose, xylose and trehalose in peptone water, ammonium salt or serum water media, as appropriate to the isolate.

The techniques were those described by Cowan and Steel (1974), except that longer periods of incubation were sometimes necessary.

Isolates identical to one or more of the phases of BLE were classified according to their growth, morphology and physiological reactions as phase A, B or C (Bisset and Bartlett, 1978).

Statistical analysis

Cultures that dried up completely or that yielded contaminants were not included in the data from the time that this occurred. Cultures that yielded BLE were recorded separately from the month after first isolation. Statistical analysis was by methods recommended by Langley (1979).

Comparisons based on the total data were made by the binomial or χ^2 tests. To compare variables on the basis of exactly comparable data, samples were arranged in pairs; each pair of samples differed for only one variable. The pairs were selected on the basis of the number of cultures put up and the length of incubation but without prior knowledge of the results, so that as many data as possible could be included in the comparison. Any data available for one sample of the pair in excess of that available for the other, e.g., additional cultures or length of incubation, were excluded from the comparison. The results obtained for strictly comparable cultures and incubation times for each pair of samples were then recorded, and the results from the sets of paired samples summarised. They were compared by the 50% probability test.

The results obtained by different groups of experimenters with pairs of cultures from the same sample were compared in the same way. The results obtained at different times of incubation were grouped within selected ranges of incubation times and compared by the binomial or χ^2 tests.

Results grouped within six-monthly ranges of incubation times were further divided into two groups for each incubation range; one group comprised the samples that gave isolates outside the selected incubation range and the other group comprised those that did not give isolates outside the range. Results for the two groups within each six-monthly incubation range were compared by the binomial or χ^2 tests. The randomness of positive results within each six-monthly range of incubation times was determined by the Runs test.

RESULTS

A total of 94 isolations of BLE were made from 11 628 subcultures of 822

cultures; 12 isolates were phase A, 15 were phase B, three were mixtures of phases A and B, and 64 were phase C. Data extracted from the results to provide valid comparisons of the different variables, and the results of statistical analysis of these data are shown in the table.

The effect of plasma on isolation rate

No significant difference was found in the number or phase of BLE isolates between cultures of whole blood and cultures of washed erythrocytes.

The effect of anticoagulant on isolation rate

No significant difference was found in the number or phase of BLE isolates between cultures made from blood collected in heparin and cultures made from blood collected in acid-citrate-dextrose anticoagulant.

The effect of different experimenters on culture results

Contamination. There was no significant difference between the number of contaminants isolated by the single experimenter and the number isolated by the group of experimenters. Sixteen contaminants were isolated from 838 cultures ($< 2\%$) within a few days of the start of incubation and generally these were assumed to have arisen from the skin of the donor; another 16 contaminants were isolated from 11 644 subcultures subsequently ($< 0.2\%$). The contaminants were 22 staphylococci, eight micrococci, one mould and one *B. cereus*.

Desiccation of cultures. Isolates subcultured by the group of experimenters dried up during incubation at a significantly higher rate than those subcultured by the single experimenter.

Isolation of BLE. No significant difference was found in the total number of BLE isolates between the single experimenter and the group of experimenters. However, the phases of the isolates differed. The single experimenter isolated phases A and B BLE more often than the group of experimenters, who isolated significantly more phase-C BLE.

The distribution of isolations of BLE with incubation time

The distribution of the isolations of the three phases of BLE with incubation time is shown in the figure. Isolations of phase A and B occurred mainly during the first year of incubation but isolations of phase C increased in frequency with incubation time. The increase in the rate of isolation of phase-C BLE with time paralleled the increase in the rate at which cultures dried up. There was no significant increase in the rate of isolation of phase-C BLE from cultures handled by the single experimenter, which had a low rate of desiccation; there was a marked increase in the rate of isolation of phase-C

TABLE
Statistical analysis of results

Criterion	Variable A	Variable B	Data compared	Number of comparable subcultures for		Number of positive events for		Probability
				Variable A	Variable B	A	B	
Isolation of BLE	Presence of plasma	Absence of plasma	Paired samples	1855	1855	14	8	> 0·10
Isolation of BLE (phase C)	Presence of plasma	Absence of plasma	Paired samples	1855	1855	6	2	> 0·10
Isolation of BLE	Heparin anticoagulant	ACD anticoagulant	Paired samples	1285	1285	14	7	> 0·10
Isolation of BLE (phase C)	Heparin anticoagulant	ACD anticoagulant	Paired samples	1285	1285	7	1	> 0·10
Contamination	Single experimenter	Group of experimenters	Total data	5198	6430	17	15	> 0·10
Drying up of cultures after incubation for 1 year	Single experimenter	Group of experimenters	Total data	1866	2452	12	82	< 0·002
Isolation of BLE	Single experimenter	Group of experimenters	Paired cultures of same samples	4200	4200	24	31	> 0·10
Isolation of BLE (phase C)	Single experimenter	Group of experimenters	Paired cultures of same samples	4200	4200	6	25	< 0·002
Isolation of BLE (phase A + phase B)	Single experimenter	Group of experimenters	Paired cultures of same samples	4200	4200	18	6	< 0·05
Isolation of BLE (phase A + phase B)	Incubation for 1 year	Incubation for > 1 year	Total data	7310	4318	27	3	< 0·01
Isolation of BLE (phase C)	Incubation for 1 year	Incubation for > 1 year	Total data	7310	4318	19	45	< 0·002
Isolation of BLE	Rheumatoid patients	Normal subjects	Paired samples	3667	3667	13	35	< 0·002
Isolation of BLE during first 6 months incubation	Rheumatoid patients	Normal subjects	Paired samples	1386	1386	4	22	< 0·002

BLE = *Bacillus licheniformis* var. *endoparasiticus*; ACD = acid-citrate-dextrose.

FIG.—The distribution of isolation of *Bacillus licheniformis* var. *endoparasiticus* with incubation time.

BLE with time from cultures handled by the group of experimenters, which had a much higher rate of desiccation.

The randomness of isolations

There was no significant difference in the rate of isolation of BLE in any 6-month incubation period between samples that gave isolates outside the period and samples that did not. Moreover, positive cultures were distributed randomly within each 6-month period of incubation after the first 6 months of incubation when there was clustering of positive cultures within samples ($p < 0.002$).

Comparison of samples from rheumatoid arthritis patients and normal subjects

Cultures from the blood of normal human subjects yielded significantly more isolates of BLE than those from the blood of rheumatoid-arthritis patients. This difference was largely due to the greater frequency of isolation from normal subjects during the first 6 months of incubation; after 6 months there was no significant difference in the number of isolates of BLE.

The proportion of different phases of BLE amongst the isolates and the distribution of the phases with incubation time were similar for the rheumatoid arthritis patients and the normal subjects.

Physiological tests on BLE isolates

The organisms isolated in the present study had the same physiological characteristics as previously described for BLE (Bisset and Bartlett, 1978).

DISCUSSION

The results show that significantly more isolates of BLE were obtained from the blood of clinically normal persons than from the blood of rheumatoid arthritis patients during the first 6 months of incubation of blood cultures. This contrasts with the findings obtained with synovial membranes and fluids by other workers who isolated "diphtheroids" (phases A and B BLE) more readily from rheumatoid synovial material (Stewart *et al.*, 1969; Duthie *et al.*, 1976). The bacteria may lodge in joints that are already diseased, as suggested by Duthie *et al.* (1976), or conditions within rheumatoid joints may affect the growth phase of the bacteria, rendering them more likely to revert to detectable forms.

After incubation for 6 months, blood cultures from normal persons and from rheumatoid patients yielded isolates of BLE at a similar rate, and isolations were randomly distributed in the cultures from different samples. This would be expected if isolation depended on random reversion from the same cell-wall-deficient form under the same conditions. The clustering of isolations in cultures of the same sample observed during the first 6 months may indicate that the L-forms in some samples are in a condition from which they are more likely to revert quickly, or that factors associated with some erythrocytes encourage early reversion. It seems that these conditions may apply more often in blood samples from normal subjects than in those from rheumatoid patients.

The isolation of BLE appears to depend on reversion of the L-form to a more readily detectable form rather than on the rate of growth of the L-form. After isolation, phase-A BLE grows much more rapidly and attains greater maximum numbers in cultures in the presence of serum or plasma; many L-forms behave similarly. However, the isolation of BLE from erythrocytes is not increased significantly by the presence of plasma.

In the later stages of incubation the results show a strong positive correlation between the isolation of the fully reverted sporing BLE (phase C) and the rate at which cultures were drying up. This correlation appeared to be the most likely cause of the different rate of isolation of phase C after prolonged incubation experienced by different experimenters. An increasing concentration of critical factors in the medium, or an increase in osmotic pressure during the slow drying of cultures, may trigger the reversion to the sporing form.

The results of this and previous work (Bisset and Bartlett, 1978) show that the L-form of BLE exists in the blood of a large proportion of human beings. It is difficult to detect by cultural methods, but after reversion to a bacterial form it grows readily on artificial media. The isolation of BLE depends on reversion of the culture, and negative cultural results after short incubation periods do

not exclude the presence of the L-form. In the present study, BLE was isolated more readily from the blood of normal subjects than from that of rheumatoid patients, whereas other workers have found that the diphtheroid phase of BLE is readily obtained from rheumatoid synovial material but not from control membranes and fluids. These findings are difficult to interpret while information about the factors affecting reversion *in vitro*, or the condition of the organism *in vivo*, is lacking. However, a difference in this respect exists between rheumatoid arthritis patients and normal subjects.

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