STUDIES OF BACTERIAL TRANSFUSION REACTIONS FROM REFRIGERATED BLOOD: THE PROPERTIES OF COLD-GROWING BACTERIA[1]

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Transfusions of blood heavily contaminated with gram negative bacilli have led to violent reactions characterized usually by irreversible hypotension and death (1–4). These reactions have resulted not only from transfusion of blood contaminated with such bacteria as the coliforms, which grow best at 37° C., but also of blood contaminated with certain unusual gram negative bacilli which are capable of better growth in the refrigerator at 4 to 8° C. than in the incubator at 37° C. Such cold-growing bacteria have long been of interest to bacteriologists dealing with problems in the fish and dairy industries, but have only recently presented an important medical problem upon being identified as contaminants in transfused human blood. Their medical importance has been emphasized by Pittman (5), Stevens, Legg, Henry, Dille, Kirby, and Finch (4), and Wetterlow, Kay, and Edsall (6).

A study of warm-growing contaminants of transfused blood has recently disclosed that 2.27 per cent of bottles in the blood bank at the University of Michigan contained bacteria which could be cultured at 37° C. (7). Reactions from these bloods, contaminated by bacteria isolated at 37° C., occurred only rarely, however, because the degree of contamination was not great enough to initiate growth in the refrigerator during storage, or at room temperature during transfusion. The bactericidal or suppressive actions of the bloods themselves were found to be important in preventing growth of contaminants at both refrigerator and room temperatures. The factors which retarded the development of these warm-growing bacteria in blood protected the recipient not only from the direct effects of the bacteria, but also from the hemolysis, increased fragility, and rare change in type, which were observed during experimental contamination of banked blood.

Because cold-growing organisms also have been held responsible for bacterial transfusion reactions, the present study was undertaken to determine their potential menace as contaminants of transfused blood. When it was found that bacteria which grow well at 4 to 8° C. could be recovered in large numbers from likely sources of contamination in the blood bank, the following points were investigated as a means of evaluating their threat to recipients of refrigerated blood:

1) The rate of growth and survival of these bacteria in refrigerated blood and in blood at room temperature.

2) The effects of their growth on the blood itself.

3) The *in vivo* toxicity of these bacteria, as determined by experimental transfusions of animals.

4) Their susceptibility to agents which might be used to suppress their growth at low temperatures.

5) Protection of animals from their toxic effects.

EXPERIMENTAL METHODS

A. *Isolation of cold-growing bacteria from sources of contamination in the blood bank and vicinity*

Three methods were used to collect bacteria which could grow at 4 to 8° C.:

1) Samples of feces, nasal discharges, air, dust, ice, soil, and snow were inoculated directly onto tryptose agar slants and incubated in the refrigerator at 4 to 8° C.

2) Materials were obtained from persons and articles in the blood bank and inoculated into banked citrated blood and tryptose phosphate broth for incubation at 4 to 8° C. Minute samples of feces, dust, nasal secretions, air, and soil were used for this purpose in an attempt to reproduce roughly the conditions under which blood might be contaminated in the blood bank.

The inoculated bloods and broths were incubated at

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4 to 8° C. and observed for growth for three weeks. Growth in blood was determined by making frequent pour plates which were incubated at both 37° C. and 4 to 8° C.

3) Secreta, excreta, and exudates from patients and hospital personnel were cultured on blood agar at 37° C. and the bacteria isolated were identified and tested for growth at 4 to 8° C. by subculturing to tryptose phosphate broth and incubating in the refrigerator. In addition to these bacteria of human origin, bacteria were also isolated at 37° C. from articles in the donor room of the blood bank and elsewhere in the hospital for study of growth in the cold.

B. *Identification of isolated bacteria*

Among the bacteria isolated at 4 to 8° C., many were coliforms which gave typical reactions, and a very few were diphtheroids and staphylococci. The great majority, however, required special means of identification. Studies on routine fermentation were conducted with the basal medium of Ayers, Rupp, and Johnson as modified by Smith, Gordon, and Clark (8).

All strains were tested for pigment production at 4°, 27°, and 37° C. on 5 per cent glycerol agar, in asparagin solution, and on tryptose agar slants containing 0.1 per cent glucose as recommended by Pittman (5). Leifson's method (9) was used for staining of flagella on all bacteria isolated in the cold.

As a means of distinguishing *Pseudomonas aeruginosa* from other pseudomonads, growth was examined at 41° C. Ability to grow at 41° C. was considered a characteristic of *P. aeruginosa* only. All bacteria isolated in the cold were incubated at 27° and 4° C. on Simmon's citrate agar, rabbit blood agar, and EMB medium.

C. *The rate of growth and survival of bacteria at cold and warm temperatures in broth and blood*

The bacteria isolated at 4 to 8° C. were compared with those isolated at 37° C. for their respective abilities to grow in the cold and at warm temperatures. A total of 102 cold-isolated bacterial strains and 65 warm-isolated strains were each inoculated into 12 ml. of recently drawn citrated banked human blood so that the concentration would be 10^3 to 10^5 viable bacteria per ml. Each strain was also inoculated simultaneously into 12.0 ml. of tryptose phosphate broth. Each sample of blood was divided into two equal parts; one sample was incubated at room temperature (27° C.) and one in the refrigerator at 4 to 8°. Growth or death in blood was measured by frequent plate counts at 27° C. Each sample of broth was divided into three equal parts; then one part was incubated at 37° C., one at 27° C., and one at 4 to 8° C. Growth in broth during 21 days was measured turbidimetrically in a Coleman colorimeter.

A second group of bloods and broth were similarly inoculated, but with heavier suspensions of 68 other bacterial strains isolated at 37° C., so that the final concentrations ranged from 10^5 to 10^8 bacteria per ml.

D. *Determination of the susceptibility of cold-growing bacteria to antibiotics in broth.*

The observation that many strains of bacterial contaminants were able to grow well in refrigerated blood led to a search for antibiotics which could inhibit their multiplication at 4 to 8° C. Antibiotics listed in Table IV were tested against large inoculums of 10^5 to 10^8 bacteria per ml. in tryptose phosphate broth; their concentrations were 2 or 20 micrograms per ml. To achieve these concentrations in banked blood, it would be necessary to add only 1 or 10 mg., respectively, to each pint of blood. These broths inoculated with bacteria listed in Table IV were divided into two samples so that one could be incubated at 4 to 8° C. and one at 37° C., unless the bacteria were incapable of growth at 37° C. Bacteria unable to produce visible growth at 37° C. were incubated at 27° C. and 4 to 8° C. At the end of 24 hours, tubes incubated at 27° C. or 37° C. were examined for growth. Tubes incubated at 4° C. were examined periodically and results were recorded when control tubes, containing no antibiotics, had reached a turbidity equal to that of control tubes incubated for 24 hours at 27° C. or 37° C., as measured in the Coleman colorimeter.

E. *Determination of the susceptibility of cold-growing bacteria to antibiotics in human blood*

Because oxytetracycline, chlortetracycline, and polymyxin B inhibited in broth more strains at 4 to 8° C. than other antibiotics, they were also examined for their ability to suppress growth in blood in the refrigerator. Twenty ml. of human blood were inoculated with the bacteria listed in Table V to give bacterial counts of 10^3 to 10^5 per ml. A plate count was made immediately after inoculation and then 18 ml. of every sample of blood was divided equally among six tubes. Two of the inoculated tubes containing 20 micrograms per ml. of antibiotic and one without antibiotic were placed in the refrigerator and the corresponding three tubes were allowed to stand at room temperature. Frequent plate counts at 27° C. were made from the bloods containing antibiotics, as well as from the controls up to three weeks.

F. *Study of the toxicity of cold-growing bacteria*

The toxicity of living bacteria in rabbits: Two experiments were conducted. In the first experiment, serial dilutions of a saline suspension of bacteria grown in tryptose phosphate broth were added directly to rabbit blood immediately before transfusion. The purpose of this was to compare the approximate minimum lethal dose in transfused blood of cold-isolated bacteria with that of ordinary coliform bacteria isolated at 37° C. The 29 strains of bacteria listed in Table VI were used. Plate counts were made immediately after addition of bacteria and the inoculated bloods were then transfused back into the corresponding rabbits through the ear vein. Rectal temperatures were taken hourly, blood was drawn from the ear vein for bacterial counts, and necropsy was carried out immediately after death. Cultures were made

of blood from the heart and from sections of liver and spleen. Surviving rabbits were sacrificed in one week.

In the second experiment, blood inoculated with sublethal numbers (10^3 to 10^5) of a given strain of bacteria was stored in the refrigerator at 4 to 8° C. for 15 days and then transfused back into the rabbit from which it had been drawn. The purpose was to determine the amount of growth in refrigerated blood necessary to produce harmful effects or death. The following bacteria were used: four strains of *A. aerogenes,* three of *E. coli,* two of *P. aeruginosa,* one of *Pr. vulgaris,* one *Pseudomonas* sp. and five of *Achromobacter* sp.

The toxicity of crude endotoxin of cold-growing bacteria in rabbits and mice: The crude endotoxin consisted of a dried bacterial residue. After incubation at 27° C. for 72 hours, the bacilli had been washed with distilled water from nutrient agar which rested on one of the four surfaces of rectangular bottles of 500 ml. capacity. The bacteria were then washed with 95 per cent ethyl alcohol twice and acetone-ether once. The residue was brought to dry weight and stored in a dessicator with calcium chloride to prevent loss of potency due to absorption of water. Prior to use, a suspension was prepared in 5 per cent sterile, pyrogen-free dextrose solution containing 20 mg. per ml. of crude endotoxin. The toxicity in rabbits of two cold-isolated strains, Achromobacter 106A and Pseudomonad 29B, was compared with that of a strain of *E. coli* isolated at 37° C. Each crude endotoxin was injected intravenously into each of six rabbits in different doses ranging from 5 to 60 mg. per Kg.

The relative toxicity in mice of the crude endotoxin of cold-isolated and warm-isolated bacteria was determined by measuring the amount of crude endotoxin required to kill in 24 hours 50 per cent of inoculated animals. Six serial dilutions of crude endotoxin, ranging in dose from 10.0 mg. to .312 mg., were each inoculated intraperitoneally into 10 white mice, weighing about 20 grams each. Each dose was given in a volume of 0.5 ml. The LD_{50} was computed by the Reed-Muench formula (10) for the crude endotoxins prepared from the 30 bacterial strains shown in Table VII.

In order to check on the possibility that variation in bacterial toxicity resulted from dilution by non-bacterial products during preparation of crude endotoxin, the nitrogen content of the crude endotoxin was determined by the micro Kjeldahl method of Koch-McMeekin. In this way, the LD_{50} could be expressed as a function of total bacterial nitrogen, as well as of bacterial weight in milligrams.

G. *Protection against the toxic effects of gram negative bacilli*

Protection of recipient animals from contaminated transfused blood: On the basis of studies with antibiotics in section E, it was decided to test the effectiveness of oxytetracycline and chlortetracycline added prophylactically to stored blood in preventing the harmful manifestations of gram negative bacilli growing in the refrigerator.

Three strains of Achromobacter and three strains of Pseudomonas, which had been isolated in the cold, were inoculated into rabbit blood which was being "banked" at 4 to 8° C. In addition, three strains of bacteria, (coliform H., coliform 2Z and coliform BCX), which had been involved in two severe transfusion reactions in human beings, were similarly added to rabbit bloods. Each bacterial strain was added to three different samples of blood which had been drawn from the heart of three individual rabbits. Oxytetracycline was added to blood from one of the three rabbits, and chlortetracycline to blood from another to give a final concentration of 20 micrograms per ml. of blood. Thus two bloods contaminated with a given bacterial strain contained antibiotics. The 27 contaminated bloods were stored for 15 days in the refrigerator at 4 to 8° C. Upon removal from the refrigerator, plate counts of the bacteria were made and then the blood was transfused back into the rabbits from which they had been originally obtained. The clinical and pathologic studies described in section F were again performed and recorded in Table VIII.

Protection against killing by crude endotoxin: Both cortisone and the two antibiotics, chlortetracycline and oxytetracycline, were examined for their ability to save animals from lethal amounts of endotoxin. Rabbits received cortisone only, and mice received cortisone and antibiotics both singly and in combination. Fifty rabbits received intravenous injections of the crude endotoxin of either *E. coli* (G), Achromobacter 101A, or Pseudomonas 29B in amounts equivalent to approximately two M.L.D.'s each as calculated in section F. The dose of toxin per Kg. in each group of rabbits is recorded in Table IX. Each rabbit was injected intramuscularly with 15 mg./Kg. of cortisone at 24 hours and one hour in advance of the injection of endotoxin, unless otherwise stated in Table IX.

The doses of crude endotoxin and the doses and time of administration of cortisone or antibiotic are listed for mice in Table X. The antibiotics and cortisone were given intramuscularly to mice in volumes of 0.1 ml. and the crude endotoxin was injected intraperitoneally in 0.5 ml. of 5 per cent glucose.

RESULTS

A. *Isolation of cold-growing bacteria from sources of contamination in the blood bank and vicinity*

Material inoculated directly onto agar slants gave rise to frequent bacterial growth at 4 to 8° C., mainly from feces, dust, and soil. The bacteria grew as distinct colonies.

Blood and broth inoculated with feces was found to contain, on the average, 10^2 to 10^3 bacteria per ml. at the time of inoculation. The great majority (90 per cent) of bloods inoculated with feces manifested heavy growth at 4 to 8° C.

Sometimes this growth represented bacteria which reached a denser population in broth at 4° C. than 37° C. These were subsequently identified as achromobacters or as pseudomonads (see Section B). Often, however, coliform bacilli, which developed to a greater density in broth at 37° C. than at 4 to 8° C. were capable of growth in blood at 4 to 8° C. About one-third of the inoculated bloods in this group showed heavy growth in three days. In the remaining two-thirds, there was no growth until two to three weeks, at which time it became heavy. One factor responsible for early growth was a larger inoculum.

Bacteria isolated at 37° C. from the secreta, excreta, and exudates of patients, hospital personnel, and articles in the donor room of the blood bank were mainly coagulase negative staphylococci, *Escherichia coli, Aerobacter aerogenes, Proteus vulgaris, P. aeruginosa, Bacillus subtilis,* and diphtheroids. The ability of these bacteria to grow in the cold is described in Section C.

B. *Identification of bacteria isolated at 4 to 8° C.*

Except for the very rare isolation of a diphtheroid or staphylococcus (coagulase negative), all bacteria isolated at 4 to 8° C. were gram negative bacilli. Among 102 strains of these gram negative bacilli, which were subjected to detailed examination, 11 were identified as *Escherichia Freundii,* 2 as *E. coli,* and 8 as coliform bacteria of intermediate type. Of the remainder, 21 were classified as members of the genus *Pseudomonas* and 60 as members of the *Achromobacter* family. None of the pseudomonads were *P. aeruginosa.* Separation of the pseudomonads from the achromobacters is difficult and not satisfactory. Achromobacters and pseudomonads possessed in common the properties of good growth in the cold, poor metabolic activity, utilization of citrate on Simmons medium, non-fermentation of lactose, and fermentation of glucose to acid only. Separation of pseudomonads from achromobacter was based mainly on the production of pigment and the location of flagellae. If an organism produced green or yellow pigment and had lophotrichous flagellae, it was considered a member of the genus *Pseudomonas.* If no pigment was produced and if flagellae were peritrichous or absent, the strain was placed in the family *Achromobacter.* Undoubtedly, some of the organisms placed in the Achromobacter family could have been regarded as members of the genus *Alkaligenes.* In the present report, however, all bacteria isolated at 4 to 8° C. which fall into the *Achromobacter* family, will be referred to as *Achromobacter* regardless of the genus.

It is of interest that the pseudomonads isolated in the cold utilized asparagine more efficiently at 4 to 8° C. than at 27° C. or 37° C. for the production of pigment. A green pigment was readily produced by them in 10 to 14 days in the cold, but only a trace, or none at all, developed during their growth at the two warmer temperatures over the same period of time. Flagellae were produced equally well at all three temperatures but were retained longer in the cold.

C. *The rate of growth and survival of bacteria at cold and warm temperatures in broth and blood*

Growth of bacteria originally isolated in the refrigerator at 4 to 8° C. from human feces, earth, dust, and air: On the basis of turbidimetric growth in broth at three different temperatures, it has been possible to classify these cold-isolated gram negative bacteria into three groups regardless of species. The composition of these groups and their composite growth curves are presented in Figure 1. Growth curves in the three groups at 4 to 8° C. are quite similar to each other. The same is true of the growth curves at 27° C. The curves at 37° C. differ from group to group and are the main basis for the classification. Bacteria in Group I produced only slight, if any, turbidity at 37° C. There was good growth at 37° C. among bacteria in Group II, even though better growth occurred at 27° C.

There is a consistent difference, also, between the slope of the curves at 4 to 8° C. and at the two higher temperatures in all groups. At 4 to 8° C., it is difficult to discern the usual phases of growth: lag, logarithmic, and stationary. Instead, there is simply a slow but gradual increase in growth density during the entire incubation period of three weeks. There is a suggestion that the logarithmic phase is greatly prolonged at 4 to 8° C. and that if incubation were prolonged a stationary phase might ultimately be reached.

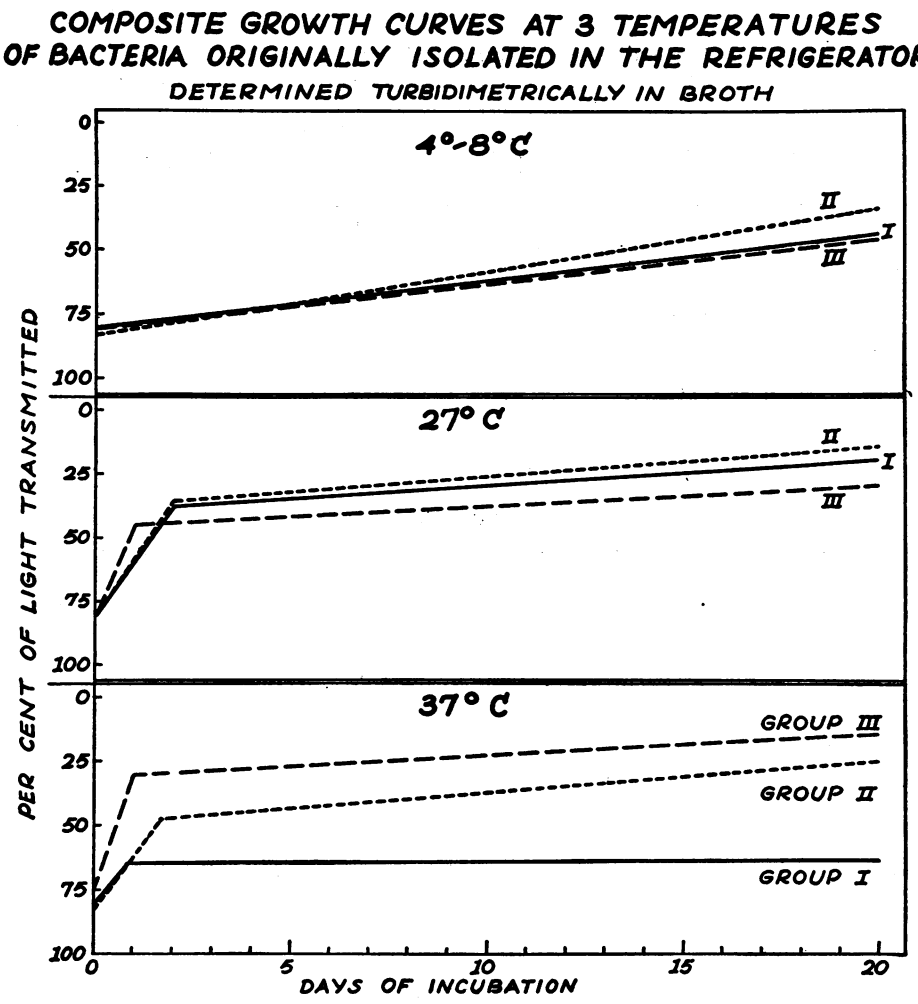

FIG. 1. CLASSIFICATION BASED ON FINAL TURBIDIMETRIC READINGS OF TOTAL GROWTH DENSITY

Group I—More growth at 4°–8° C. than 37° C. and most at 27° C.:
28 Achromobacters, 4 Pseudomonads.

Group II—Most growth at 27° C. More growth at 37° C. than 4°–8° C.:
11 Achromobacters, 10 Pseudomonads, 2 *E. Freundii.*

Group III—Most growth at 37° C.:
21 Achromobacters (mainly Alcaligenes), 21 Coliforms (11 *E. Freundii,* 2 *E. Coli,* 8 Intermediate Coliform), 5 Pseudomonads.

A composite curve is made by combining the individual growth curves for each of the bacterial strains in a group.

The three phases of growth are clearly defined when incubation in broth is carried out at 27° C.

On the basis of this classification, the growth and survival of these cold-isolated bacteria in blood and broth may be summarized as shown in Table I when small inoculums (10^2 and 10^3 viable cells per ml.) are used. The results may be compared to those summarized in Table II, which were obtained with small inoculums (10^2 and 10^3) of bacteria originally isolated at 37° C.; and with those summarized in Table III, which were obtained with large inoculums (10^5 to 10^8) of bacteria originally isolated at 37° C.

Among bacteria originally isolated in the cold,

TABLE I

Growth and survival of bacteria originally isolated at 4° C.

Group	Characteristics of total growth as measured by cultural density in broth	Total number of strains	Number showing growth in blood		Number showing growth in broth		
			Temperature of growth				
			4° C.	27° C.	4° C.	27° C.	37° C.
1	More growth at 4° C. than 37° C. Most growth at 27° C.	32	15	14	32	32	21
2	More growth at 37° C. than 4° C. Most growth at 27° C.	23	17	10	23	23	23
3	Most growth at 37° C.	47	37	33	47	47	47
	Totals	102	69	57	102	102	91

TABLE II

Growth of small inoculums (10^2 to 10^3) of bacteria originally isolated at 37° C.

Species	Total number of strains	Number showing growth in blood		Number showing growth in broth	
		Temperature of growth			
		4° C.	27° C.	4° C.	27° C.
Staphylococcus (coagulase-negative)	8	0	7	0	8
E. coli	21	6	11	0	21
A. aerogenes	11	3	8	0	11
Pr. vulgaris	13	3	13	1	13
P. aeruginosa	6	1	6	0	6
Paracolon sp.	4	3	1	2	4
B. subtilis	2	0	2	0	2
Totals	65	16	48	3	65

TABLE III

Growth of large inoculums (10^5 to 10^8 per ml.) of bacteria originally isolated at 37° C.

Species	Total number of bacterial strains	Number showing growth in blood			Number showing growth in broth		
		Temperature of growth					
		4° C.	27° C.	37° C.	4° C.	27° C.	37° C.
Staphylococcus (coagulase-negative)	22	6	20	20	10	22	22
E. coli	16	6	12	12	14	16	16
A. aerogenes	6	2	5	5	6	6	6
Pr. vulgaris	10	9	9	9	10	10	10
Paracolon sp.	5	4	5	5	5	5	5
P. aeruginosa	3	1	3	3	3	3	3
B. subtilis	4	2	2	2	Not tested	4	4
Diphtheroids	2	2	2	2	1	2	2
Totals	68	32	58	58	49	68	68

about 70 per cent grew in blood and 100 per cent grew in broth at 4 to 8° C. from small inoculums. These results are in contrast to those summarized in Table II which illustrate that growth in blood at 4 to 8° C., among small inoculums of bacteria isolated at 37° C. was not common (25 per cent) and growth in broth with these inoculums was very unusual (5 per cent).

Of the 70 per cent isolated in the cold, which grew well in blood stored at 4 to 8° C., many strains grew heavily in about one week and most by two weeks. They had often reached concentrations of 10^8 to 10^{10} bacteria per ml. by then. At refrigerator temperatures, these bacteria produced hemolysis in about 75 per cent of the bloods and clotting in about 15 per cent. The clotting was due to the consumption of citrate (11). Blood type was never changed in the cold.

Those strains isolated in the cold, which grew well at 37° C. in broth (Groups II and III), more frequently multiplied in blood at 4 to 8° C. than those that grew poorly in broth at 37° C. (Group I). Eight strains of Group I, including pseudomonads and achromobacters, which grew at 27° and 4 to 8° C., failed to grow visibly in broth at 37° C. There is a possibility that such bacteria might be missed if contaminated blood were cultured at 37° C. only. Two of these eight strains grew heavily in blood at 4° C.

Small inoculums of the cold-isolated bacteria did not grow as frequently in blood when incubated at 27° C. as did those bacteria isolated at 37° C. When growth did not occur at 4 to 8° C. or 27° C. in blood, the number of bacteria diminished markedly.

Growth of bacteria originally isolated at 37° C.: With the smaller inoculums of warm-isolated bacteria, growth could seldom be measured turbidimetrically with the colorimeter when the bacteria had been incubated in broth at 4 to 8° C. for three weeks. Only 16 of the 64 strains grew in blood in three weeks at 4 to 8° C. and never as heavily as

TABLE IV

Inhibition of bacterial contaminants by antibiotics at 37° C. and 4° C. in broth *

	Bacteria	Total no. of strains	Concentration of antibiotic *mcg./ml.*	Temperature of incubation during exposure to antibiotics: 4° C. — Number of strains whose growth was prevented: C	AU	P	T	S	AE	37° C. (or 27° C.) — Number of strains whose growth was prevented: C	AU	P	T	S	AE
	Pseudomonads	15	2	2	7	2	5	3	8	1	1	1	4	4	7
	(not *P. aeruginosa*)	11	20	2	8	2	8	6	7	6	6	1	7	9	8
	Achromobacter	28	2	2	11	1	3	4	12	5	6	1	5	7	13
		14	20	3	9	1	6	5	8	8	3	2	4	8	10
Isolated at 4° C.	Coliforms	11	2	1	2	0	2	2	3	0	1	1	1	0	1
		6	20	5	6	1	6	5	5	3	2	1	4	1	3
	Diphtheroids	4	2	2	3	2	2	0	2	3	2	3	3	2	3
		4	20	2	3	2	3	2	4	3	4	3	3	3	3
	Total of those isolated at 4° C.	58	2	7	23	5	12	9	25	9	10	6	13	13	24
		35	20	12	26	6	23	18	24	20	15	7	18	21	24
	Proteus	6	2	2	1	0	3	2	3	0	1	0	0	3	2
		6	20	3	4	1	4	3	3	2	3	0	2	0	2
	E. coli	10	2	1	2	3	4	3	7	0	4	1	1	0	8
		10	20	3	4	5	5	3	6	10	7	2	7	7	7
Isolated at 37° C.	*A. aerogenes*	3	2	1	1	1	1	2	3	0	1	0	0	1	2
		3	20	2	2	1	1	2	3	3	3	0	3	3	3
	Coliforms	5	2	1	1	0	2	1	1	1	2	1	2	0	1
		5	20	1	1	0	3	2	2	4	3	1	4	2	2
	Total of those isolated at 37° C.	24	2	5	5	4	10	8	14	1	8	2	3	4	13
		24	20	9	11	7	13	10	14	19	16	3	16	12	14
	Total of all strains capable of growth at 4° C.	82	2	12	28	9	22	19	39	10	18	8	16	17	37
		59	20	21	37	13	36	28	38	39	31	10	34	33	38

* C = chloramphenicol; Au = chlortetracycline; P = penicillin; T = oxytetracycline; S = streptomycin; Ae = polymyxin B (aerosporin).

TABLE V

Inhibition of large bacterial inoculums in blood by tetracycline antibiotics in concentrations of 20 mcg./ml.

		Temp. (*degrees Centigrade*)	Total strains	Number of strains whose growth was prevented in blood		
				No antibiotics	Chlortetracyclene	Oxytetracyclene
Isolated at 4° C.	Achromobacter	4	26	8	25	25
		27	23	10	20	22
	Pseudomonads	4	17	2	16	17
		27	17	13	17	17
	Coliforms	4	16	0	11	13
		27	14	2	9	13
	Totals	4	59	10	52	55
		27	54	25	46	52
	E. coli	4	9	5	9	9
		27	10	5	10	10
	A. aerogenes	4	6	5	6	6
		27	6	2	6	5
Isolated at 37° C.	*Pr. vulgaris*	4	15	11	15	15
		27	5	0	5	5
	Intermediate Coliforms	4	2	0	2	2
		27	3	3	3	3
	Totals	4	32	21	32	32
		27	24	10	24	23
	Overall Totals	4	91	31	84	87
		27	78	35	70	75

cold-isolated bacilli. Nearly all strains of coagulase negative staphylococcus, *E. coli, Pr. vulgaris,* and *A. aerogenes* were killed in blood after three weeks in the refrigerator. Of the 16 organisms which grew at 4 to 8° C. in blood, 5 produced hemolysis. At 27° C., growth in blood was nearly as frequent with the smaller inoculums as with the larger.

With the larger inoculums of warm-isolated bacteria, growth in the refrigerator was about twice as frequent as with the smaller inoculums. Thus, of the 68 strains inoculated heavily, 32 grew at 4 to 8° C. in blood and usually reached numbers which would probably be lethal upon transfusion. Nearly all strains grew in blood eventually at room temperature or in the incubator. Frequently, an initial reduction in plate counts was observed during the first few days of incubation at 27° and 37° C. Proteus grew fastest at all three temperatures of all warm-isolated species tested in both blood and broth. Coagulase negative staphylococci, *E. coli,* and *A. aerogenes* did not frequently grow and most of the strains of these species died in blood in the refrigerator. Nearly all of these gram negative bacilli produced visible growth in broth in the refrigerator. On the other hand, only 10 of 22 strains of a staphylococci grew visibly in broth in the refrigerator. It would appear, therefore, that blood suppressed growth or killed large inoculums of *E. coli, A. aerogenes,* and *P. aeruginosa* in the refrigerator since almost all of them could grow in broth at 4 to 8° C. Staphylococcus, on the other hand, frequently could not grow at 4 to 8° C. regardless of the media, so that the bactericidal or bacteriostatic action of blood was not a necessary factor for restricting development of that species in the refrigerator.

D. *The susceptibility of cold-growing bacteria to antibiotics in broth*

The results summarized in Table IV indicate that at 4 to 8° C. oxytetracycline, chlortetracycline, and polymyxin B were the most effective of the antibiotics. Chlortetracycline, an unstable antibiotic at 37° C., was more effective in the cold.

There was no difference between the effectiveness of polymyxin B at cold and warm temperatures. The higher of the two concentrations of chloramphenicol was less effective at cold temperatures than at warm temperatures.

E. *The susceptibility of cold-growing bacteria to antibiotics in blood*

The results are summarized for the two tetracycline antibiotics in Table V. Results indicate whether or not there was inhibition of growth in three weeks for refrigerated blood and at 36 hours for blood stored at 27° C.

Blood containing chlortetracycline almost never permitted growth at 4 to 8° C. Chlortetracycline occasionally permitted growth of cold-isolated bacteria (15 per cent) at 27° C. but not of warm-isolated bacteria. Oxytetracycline almost never permitted growth at either temperature and polymyxin B, tested only for its effectiveness in the cold, suppressed 92 per cent of bacteria. When no growth occurred in bloods containing antibiotics, the bacteria were usually reduced in number and sometimes sterilized in three weeks or less.

F. *The toxicity of cold-growing bacteria*

The toxicity of living bacteria in rabbits: The results are summarized in Table VI. The maximum numbers of *Achromobacter* or *Pseudomonas* which could be given in transfused blood without

TABLE VI

Toxicity (acute pathogenicity) of living gram negative bacilli for rabbits when transfused in 7.0 ml. of homologous rabbit blood *

Bacteria	Maximum sublethal dose	Maximum fever of any rabbit (*degrees Fahrenheit*)	Bacteria per ml. rabbit blood at 3 hours		Pathologic changes	
			Lethal dose	Sublethal dose	From lethal dose	From sublethal dose
A. aerogenes 3	10^9	4.5	I	3,960	0	NRT; MHA
A. aerogenes 1	10^9	2.8	862	3	0	PA; NRT
A. aerogenes 2	10^9	3.5	21,600	320–2,000	GT; NRT	HA; PA
E. coli 1	10^8	2.6			MHA	
E. coli 3	10^8	4.8		5–20	0	NRT; FRT
E. coli 2	10^7	3.4	2,500	47	NRT	
E. coli 4	10^8	3.5	510	13	NRT	NRT
P. aeruginosa A	10^{10}	4.0	I	164	GT	PA; HA
P. aeruginosa B	10^9	4.0	192	2–28	0	0
P. aeruginosa 1	10^9	3.9	306	6–220	0	NRT
P. aeruginosa C	10^7	4.0	49	6	0	0
Pr. vulgaris 11	10^8	4.0			FNS	0
Pr. vulgaris 19 A	10^8	4.1	2,000			
Pr. vulgaris R	10^9	4.3	3,665	88	NRT	0
Pr. vulgaris A	10^8	4.4	150	0	0	0
Coliform Hunt	10^{10}	4.4		200		
Coliform Bcx	10^8	4.4	104	12		
E. Freundii G	10^8		320	0		
Coliform G 2X	10^8	4.2		0–5		
Achromobacter 23	10^9	3.7	126	13–26		
Achromobacter 13A	10^{10}	2.5	3			HA
Achromobacter 9	10^8	4.0	810	7–134	MHA	FNS
Achromobacter SC	10^9	3.5		5		NRT
Achromobacter 250E	10^9	4.1	520	3–21	0	
Pseudomonad 29B	10^9	5.8	4	0	0	
Pseudomonad 20A	10^8	3.7	3	2	FNS	NRT
Pseudomonad 30A	10^{10}	4.4		330		
Pseudomonad 17B	10^{10}	4.5		152		
Pseudomonad 14B	10^8	5.0	168	40–60	FNS	

* Bacteria were added to blood immediately before transfusion. Living rabbits sacrificed at one week.
NRT = necrosis renal tubules; PA = pulmonary abscess; HA = hepatic abscess; M = miliary; FNS = focal necrosis of spleen; GT = glomerular thrombi; I = innumerable.

Dr. C. V. Weller assisted the authors in making these histopathologic interpretations.

TABLE VII

L.D./50 of crude bacterial endotoxins for mice (milligrams)

Isolated at 37° C.			Isolated at 4° C.		
P. aeruginosa	21	1.48	Pseudomonad	(dust) 14	1.11
	A	.417		(dust) 30A	1.45
	P	.417		(feces) 29B	.884
	B	.264		(dust) 20A	.845
E. coli	G	.368	Achromobacter	(feces) 106A	.312
	4	.361		(feces) 12C	1.87
	2	.350		208A	.675
	C	.557		(feces) 13A	1.85
				(feces) 23	.992
A. aerogenes	1	.061		(feces) 81	1.40
	N	.836			
	B	.739			
	3	.999			
Pr. vulgaris	B	1.53		Mean	1.14
	M	.850			
	S	.884			
	11	1.32			
Intermediate Coliform	H	1.52			
	2	1.65			
	B	.981			
	3	.661			
Mean		.843			

killing rabbits ranged from 10^8 to 10^{10} bacteria. The maximum sublethal numbers of coli-aerogenes, *P. aeruginosa,* and *Pr. vulgaris* were about the same. Lethal and sublethal numbers of the cold-isolated bacteria, pseudomonads, and achromobacters produced changes similar to those seen in animals receiving strains of coli-aerogenes, *P. aeruginosa,* and *Pr. vulgaris.* These consisted of necrosis of renal tubular epithelium, focal necrosis of the spleen, and generalized abscess formation. Many animals were free of pathologic changes. It is of interest that bacteremias at 3 hours in dying animals were more intense after transfusion of the warm-isolated bacteria than of the achromobacters and pseudomonads.

The chief findings in dying animals after transfusion with contaminated blood were severe weakness, fever, diarrhea, and a marked tendency to bleeding from the intestine and elsewhere. These findings were nearly identical in rabbits transfused with contaminated blood regardless of whether the contaminants were cold-growing or warm-growing bacteria.

The amount of growth in refrigerated blood required for lethal numbers of bacteria to develop from sublethal inoculums of cold-isolated and warm-isolated bacteria was to approximately 10^8 to 10^{10}, or the same as the lethal numbers after suspension of the bacteria in blood immediately prior to transfusion. Hence, it would appear that the toxic effects of transfused blood may depend on the number of gram negative bacilli; and that by-products of growth may not seriously influence the total toxic effect.

The toxicity of crude endotoxin in rabbits and mice: Various amounts of crude endotoxin from an achromobacter and a pseudomonad, both isolated at 4 to 8° C. produced essentially the same toxic effects in rabbits as equal doses of crude endotoxin prepared from a strain of *E. coli.* Doses of 10 to 15 mg. per Kg. of any of the three toxins killed all rabbits tested in 3 to 20 hours. Before death the rabbits displayed fever, diarrhea, weakness, and rectal bleeding. Doses of 5 mg. per Kg. or less led to diarrhea, listlessness, and fever but the rabbits survived.

In Table VII, the amount of crude endotoxin required to kill in 24 hours 50 per cent of inoculated mice is given for large numbers of cold-growing and warm-growing gram negative bacilli. The results indicate that the cold-isolated bacteria are nearly as toxic for mice and rabbits as the common enteric bacteria.

The total bacterial nitrogen concentration va-

ried from .10 to .14 mg. per mg. of crude endotoxin. The constancy of bacterial nitrogen from strain to strain indicates that equivalent amounts of bacterial products were being compared and that variations in toxic activity did not result from dilution of some endotoxins by non-bacterial products. Instead, the variations in toxicity would be expected to result from differences in potency of a given endotoxin, or from differences in total bacterial content of endotoxin.

G. *Protection against the toxic effects of gram negative bacilli*

Protection of recipient animals from contaminated transfused blood: The results in Table VIII disclose that the presence of antibiotics suppressed or destroyed the contaminants in transfused blood and completely prevented the bacteremia and severe *in vivo* effects observed in the controls. All control animals, receiving contaminated bloods without antibiotics, suffered fatal or nearly fatal reactions.

Protection by cortisone and antibiotics against crude endotoxin: In Table IX are the results of treating rabbits with cortisone after inoculation of lethal amounts of crude endotoxin from a pseudomonad, an achromobacter and a strain of *E. coli.* No protection by cortisone was observed among rabbits given *E. coli* toxin, but 14 of 15 cortisone treated rabbits survived the toxic effects of the two other toxins given in amounts which killed 13 of 15 controls. It is concluded that cortisone protected rabbits from the lethal effects of the crude toxins prepared from the two cold-growing bacteria.

TABLE VIII

Protection against bacterial transfusion reactions by antibiotics in contaminated blood (20 mcg./ml.) transfused into rabbits after 15 days refrigeration *

Bacteria	Total number of bacteria: Original inoculum	Total number of bacteria: Given in transfusion	Blood culture *Bacteria/ml.*	Antibiotic	Outcome
Coliform 2Z	10^4	10^{10}	—	None	Died 12 hrs.
		10	0	A	Normal
		10	0	T	Normal
Coliform BCX	10^4	10^{10}	I	None	Died
		10^3	0	A	Normal
		10^3	0	T	Normal
Coliform H	10^4	10^7	—	None	Died 5 hrs.
		0	0	A	Normal
		0	0	T	Normal
Achromobacter 81	10^5	10^8	91	None	Died 12 hrs.
		10^2	0	A	Normal
		10^4	0	T	Normal
Achromobacter 23	10^5	10^{10}	I	None	Died 10 hrs.
		10^4	0	A	Normal
		10^4	0	T	Normal
Achromobacter 9	10^5	10^{10}	2,970	None	Died 12 hrs.
		10^5	0	A	Normal
		10^5	0	T	Normal
Pseudomonad 29B	10^6	10^{10}	100	None	Died 20 hrs.
		10^7	0	A	Listless
		10^2	0	T	Normal
Pseudomonad 14B	10^5	10^8	12	None	Sick 3 days
		0	0	A	Normal
		10^3	0	T	Normal
Pseudomonad 20A	10^5	10^{10}	106	None	Died 4th day
		10^5	0	A	Normal
		10^3	0	T	Normal

* A = chlortetracycline, T = oxytetracycline, I = innumerable.

TABLE IX

Results treating rabbits with intramuscular cortisone (15 mg./Kg.) at 24 hours and again at one hour in advance of intravenous injection of crude endotoxin (approximately 2 m.l.d.)

Crude toxin	Dose of toxin (mg./Kg.)	Number of rabbits surviving up to 24 hrs.	
		Treated	Not treated
Pseudomonad 29B	20	5/5*	0/5
	15	5/5	2/5
E. coli G	12.5	0/5	0/5
	16	0/5†	0/5
Achromobacter 106A	25	4/5	0/5

* Received additional cortisone (15 mg./Kg.) 48 hours before toxin.
† Only received cortisone one hour before toxin.

In Table X, similar protection against crude endotoxin is seen in mice pretreated with cortisone but not in mice receiving cortisone at the same time the toxin was injected or afterwards. There is a suggestion in Table X that the tetracycline antibiotics also provided protection when given in advance of endotoxin. There was no sign of protection when the antibiotics were given at the same time, or after, the crude toxin.

DISCUSSION

Each of the two types of bacterial contaminants of banked blood presents a separate problem. First there are the cold-growing contaminants. These seem to be remarkably well suited for endangering the recipients of banked blood. Not only do they inhabit the premises and personnel of a blood bank, but they resist the bacteriostatic action of blood to such an extent that even small inoculums multiply heavily in the refrigerator, and sometimes at room temperature. These cold-growers are gram negative bacilli whose toxic potency is nearly equal to that of the endotoxins of the common intestinal gram negative bacilli.

The second type, which are classified as warm-growing contaminants, have less ability to grow in refrigerated blood, but grow better in blood at room temperature than the cold-growers. In Table XI, which lists the bacteria responsible for the violent transfusion reactions from contaminated blood reported to date, there are eleven, which are probably warm-growers, and seven, which would be expected to be cold-growers according to the present definition. One of the strains of *E. freundii* might have fallen into either group. These reports, therefore, suggest that the two types of contaminants are of nearly equal importance in leading to bacterial transfusion reactions.

Once blood becomes contaminated, the possibility of a serious reaction is not great because most contaminants are non-toxic gram positive bacteria which do not readily survive in refrigerated blood (7). Reactions will occur only when gram negative bacteria are stored in the cold or stand at room temperatures long enough

TABLE X

Protection of mice from lethal effect of crude endotoxin

Crude endotoxin	Dose (mg.)	Mortality rate in 24 hours			
			Cortisone 5 mg.		Antibiotics* 2 mg.
		Controls	8 hrs. before toxin	With toxin or later	8 hrs. before toxin
E. coli 4	.8	9/20	0/20		
	1.6	55/60	3/20	57/60	
A. aerogenes 3	5.0	20/20		20/20	
	2.5	36/40	7/20	34/40	11/20 (A)
Pr. vulgaris 11	5.0	20/20	8/20	17/20	6/20 (T)
P. aeruginosa	1.0	19/20	6/20		5/20 (A)
Achromobacter 13A	1.0	11/20	3/20		9/20 (A)
Totals		170/200	27/120	128/140	31/80

* A = chlortetracycline; T = oxytetracycline.

TABLE XI

Identification of bacteria reported to have been associated with severe bacterial transfusion reactions; of the 19 strains, 4 were said to be incapable of growth at 37° C. (Pseudomonas [5], [4])

Number of reactions associated with each bacterial species		Reported by
E. coli	3	Pittman (5); Officer (15)
"B. coli aerogenoides"	4	Whitby (2)
E. freundii	2	Braude *et al.* (3); Pittman (5)
Paracolon aerogenoides	2	Borden and Hall (1); Pittman (5)
Alcaligenes fecalis	1	Pittman (5)
Achromobacter sp.	1	Borden and Hall (1)
Pseudomonas sp.	5	Whitby (2); Pittman (5); Stevens *et al.* (4)
Intermediate Coliform	1	Braude *et al.* (3)
Total	19	

to grow to toxic proportions. It is likely that heavy growth at cold temperatures would result after contamination of banked blood with either a few cold-growers or numerous warm-growers, or at room temperature with small numbers of either type of gram negative contaminants. Precise limits on the period of storage or standing which would avoid heavy growth in contaminated blood cannot be given. It has been established in the present experiments, however, that the logarithmic growth phase is decelerated at cold temperatures so that a week or more is required for heavy bacterial growth in refrigerated blood. At room temperature, on the other hand, the typical steep logarithmic phase begins in about 6 hours in blood containing the usual inoculum of both cold and warm-growers, and by 24 hours, growth usually reaches lethal proportions (7). From these observations, it is clear that a minimum of standing in the refrigerator or donor room will reduce the hazard of bacterial reactions. Blood from banks with a turnover of most bottles within a few days should threaten fewer recipients with bacterial reactions than blood from banks which keep most bottles for more than one week. If a hospital cannot arrange for all bloods to be examined for bacterial contamination by direct smear at the time of transfusion, it should do so at least for those bloods refrigerated for longer than one week.

There is a possibility that gram negative bacilli may be so numerous at the time of contamination that their growth is unnecessary for the blood to produce a violent reaction. Reactions from such heavy contaminations have in fact been observed experimentally (7) and suspected clinically (3). They would be due to gross uncleanliness and negligence and could hardly be prevented by any known precaution once contamination had occurred. The only form of contamination from which the recipient can be confidently protected by precautionary measures is that in which growth is still required for the blood to assume dangerous properties. The routine use of either chlortetracycline, oxytetracycline, or polymyxin B in concentrations of 10 mg. per pint can prevent large sublethal inoculums of either cold-growers or warm-growers from multiplying in human blood at both refrigerator and room temperature (Table V). Transfusion of contaminated blood containing that concentration of chlortetracycline or oxytetracycline during refrigeration produced no harm to animals under conditions which resulted in the death of nearly all control animals (Table VIII). Such minute quantities of either tetracycline antibiotic would probably be harmless to the recipients. Patients who receive blood transfusions are frequently given a variety of antibiotics for prophylactic and therapeutic reasons. The small amount in the transfused blood would be negligible compared to what he is likely to be receiving otherwise. The routine use of the tetracycline antibiotics in all banked blood has been suggested by Stevens and his co-workers (4) and should be seriously considered especially under those difficult conditions of storage or handling which may be encountered in civil catastrophes or war. Blood alone is bacteriostatic against only some of the contaminants (Table V); the same is true of antibiotics (Table IV). But together they are remarkably effective in preventing growth of nearly all warm-growing or cold-growing types of contaminants. It is of interest that the tetracycline antibiotics have also proved the most effective inhibitors of growth of the natural mixed bacterial flora of fish and meat stored at temperatures between 0° C. and 21° C. (12).

If growth of gram negative bacilli is not prevented before transfusion, they may kill the recipient. The mechanism of killing is related to the action of the bacterial endotoxin and not necessarily to invasive properties of the bacteria. Thus, rabbits transfused with lethal numbers of cold-growing bacilli (pseudomonads or achromobacters) had sometimes cleared their blood al-

most entirely of bacteria at the time of death (Table VI). These animals had apparently died of the effects of endotoxin rather than those of an overwhelming septicemia resulting from *in vivo* growth of the bacteria. Similar clinical phenomena after transfusions of heavily contaminated blood have been seen in patients who were in profound shock or died despite the absence of demonstrable bacteria in the blood or tissues (1, 3, 4). The chief source of toxin from gram negative bacilli multiplying in refrigerated blood appears to be the endotoxin within the bacilli; other toxic products are apparently not manufactured in significant amounts during growth in blood. This conclusion is based on the observation in this study that lethal numbers of viable bacteria, resulting from multiplication in blood, are approximately the same as lethal numbers after suspension of bacteria in blood immediately prior to transfusion. Geller and Jawetz (13) have recently presented evidence that in mice part of the bacterial multiplication involved in reaching lethal doses of endotoxin may take place *in vivo*.

Once transfusion of heavily contaminated blood has occurred, it is difficult to provide protection and the recipient usually dies. In experimental animals challenged with endotoxin, the most consistently effective protective measure has been prior treatment with cortisone. In these studies, protection from cortisone was observed not only in rabbits given crude endotoxin obtained from cultures of *Achromobacter* and pseudomonads, but also in mice challenged with crude endotoxins of *E. coli, A. aerogenes, Pr. vulgaris, P. aeruginosa,* and *Achromobacter*. Similar observations have been reported earlier by Boyer and Chedid (14). Antibiotic treatment of animals given crude endotoxin was less consistently effective in these experiments than treatment with cortisone. In the only report of successful treatment of an overwhelming bacterial transfusion reaction, the patient received not only antibiotics and cortisone, but nor-epinephrine as well (3). In fact, the use of nor-epinephrine in large quantities for several days appeared to be the most valuable measure of all those employed in the successful treatment. This drug was essential for overcoming the state of severe and persistent hypotension which is the most striking clinical effect of transfusing contaminated blood.

Perhaps the crucial point in establishing successful treatment is prompt recognition of the syndrome. Unfortunately the patient who has received contaminated blood may not appear gravely ill during the first 12 hours after the transfusion and there may be few outspoken signs to call attention to the serious state of hypotension. The syndrome is most quickly identified by examining a direct smear of the blood from the transfusion bottle. Gram negative bacilli will be found in every field, and treatment must be instituted at once without waiting for identification of the bacteria.

When attempts are made to recover the contaminating bacteria, cultures should be incubated at 27° C. as well as 37° C. because an occasional strain of cold-growing bacteria cannot grow at the usual incubator temperatures. All but 4 of the 19 reported strains listed in Table XI could be cultivated at 37° C. These organisms, which cannot grow at 37° C., represent only a small minority of cold-growing bacteria and are less capable of vigorous multiplication in refrigerated blood than those cold-growing bacteria which can multiply well at 37° C. It is not surprising, therefore, that they have been incriminated only infrequently as the cause of transfusion reactions.

The cold-growing bacterial contaminant of blood must be regarded as a newly recognized type of pathogenic microorganism. It is pathogenic because of its capacity to produce both clinical and experimental disease; but lacking any significant ability to multiply in the host, it exerts its pathogenicity entirely through an endotoxin which is produced outside of the body and injected intravenously. The vector is thus man himself who confers upon the bacteria the attribute of communicability. The cold-growing bacteria may be compared in this sense with *Clostridium botulinum* which also lacks invasive capacities and depends entirely for its pathogenicity on toxic properties. The great point of difference between the two microorganisms, however, is that the toxicity of *Cl. botulinum* is due only to its potent exotoxin. It possesses no endotoxin.

SUMMARY

Bacteria capable of heavy growth at refrigerator temperatures (4 to 8° C.) can be easily re-

covered in the blood bank from potential sources of contamination for stored blood. The majority of these cold-growing contaminants are gram negative bacilli which have been identified as pseudomonads, coliforms, and achromobacters. Their toxic potency is nearly equal to that of the endotoxins of common intestinal bacilli. Because even small inoculums can resist the bacteriostatic action of blood and multiply quickly to lethal numbers in the refrigerator, these cold-growing bacilli constitute a grave threat to recipients of contaminated banked blood. They may be consistently suppressed, however, in blood containing such minute amounts of tetracycline antibiotics that risk to the recipient from the antibiotics is unlikely.

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