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WHOLE BLOOD AMINO ACID CHANGES FOLLOWING RESPIRATORY-ACQUIRED *PASTEURELLA TULARENSIS* INFECTION IN MAN

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The importance of amino acids in nutrition and in intermediary metabolism has been studied extensively. Few studies, however, have been performed to evaluate amino acid changes in terms of the response of man to infection.

Disturbances of amino acid and nitrogen metabolism have been demonstrated in rats during infection with *Pasteurella tularensis* (Woodward et al, 1954; Sbarra and Woodward, 1955; Woodward and Miraglia, 1964). The only controlled metabolic studies of *P. tularensis* in man are those of Beisel (1965) who reported changes in the urinary excretion of urea, creatinine, ammonia, uric acid and alpha-amino nitrogen. Determinations of circulating amino acids were not performed. As part of an attempt to survey and correlate human responses to infection, whole blood amino acids were measured in volunteers following aerosol exposure to *P. tularensis*.

METHODS

Amino acid determinations were performed by the technique of Efron et al (1964) which permits the analysis of normally circulating amino acids in 6 microliters of whole blood. The procedure was modified as follows: Ninhydrin-stained chromatograms were dried and developed in a chromatography oven at 110 C for 8 minutes. Each chromatogram was quantitated by densitometric analysis with an Analytrol (model RB, Beckman Instruments, Inc., Palo Alto, California) to obtain an integrated tracing. The integrated value for each amino acid detectable as a single spot including cystine, alanine, alpha-amino butyric, tyrosine, and phenylalanine was converted to micrograms per 0.006 ml by comparison with a standard curve prepared for each amino acid. Thus, single amino acids can be reported in mg per 100 ml by use of the appropriate multiplication factor.

The summation of the integrated values for each singly identified amino acid, as well as values obtained for amino acid groups, gave a total value that has been equated with the term total amino acids. Groups containing leucine and isoleucine; valine, methionine, and tryptophan; glutamic acid and threonine; glycine, serine, glutamine, and taurine; and histidine, lysine, and arginine have been included. Concurrent analysis of as many as 60 samples per chromatography tank permitted the simultaneous analysis of all samples obtained from any subject and minimized changes attributable to methodological variables. The error of the method calculated from 3 standard deviations of the mean total integrated value of 20 replicate determinations of the same blood sample on the same day was 2.1%. The error of the method calculated similarly from replicate de-

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terminations on the same blood sample on 20 different days was 5.7%.

Eighteen healthy young males, aged 19 to 26 years and participating on a voluntary basis, were employed for this study. They were fully informed of the nature and details (including risk) of the studies prior to participation† (U. S. Army Regulation, 1962). Prior to exposure, each subject was ascertained to be in excellent health by careful history, physical examination, and numerous laboratory determinations. Sixteen individuals inhaled approximately 25,000 viable organisms from a dynamic aerosol (Henderson, 1952) of *P. tularensis* at 9 A.M. on day 0. The remaining 2 subjects were sham-exposed. Blood was obtained by venipuncture before exposure at 8 A.M., then at 2 and 8 P.M., and thereafter at 8 A.M. and 8 P.M. daily. Rectal temperatures were taken every 6 hours from the time of admission until discharge. Pharyngeal washes for *P. tularensis* were plated on solid media (Gaspar et al, 1961). Serological tests were performed 1 week before exposure and 2, 4 and 7 weeks after exposure (Technical Manual 8-227-1, 1960).

Following exposure of the subjects, signs and symptoms of each, exclusive of fever, were evaluated and rated on a 1 through 4 scale. Fever responses of each individual were evaluated separately and assigned 2 ratings representative of height and duration. The 3 values obtained for each subject were multiplied and the product defined as the illness index.

Therapy (Sawyer et al, 1966) with orally administered tetracycline (1 g every 6 hours for 4 doses followed by 500 mg every 6 hours for 14 days) was initiated when a clinical rating of 2 or 3 was noted in the presence of fever of 101 F or when fever of 102 F or greater was sustained for 24 hours even in the absence of other signs or symptoms. If none of the criteria were fulfilled but a period of time greater than 15 days from the day of exposure had elapsed, therapy with streptomycin (1 g every 12 hours for 14 doses) was initiated.

Sham-exposed individuals received similar courses of tetracycline begun on day 5.

RESULTS

The 8 A.M. amino acid concentration of each subject during the control period has been equated with an amino acid level of 100%. The reported percentage increases or decreases from control levels have been calculated with subsequent 8 A.M. samples. In this manner, each subject has served as his own control and been compared with sham-exposed subjects.

Temperature and amino acid concentrations of the 2 noninfected individuals (cases 1 and 2) remained within normal limits throughout the period of hospitalization, as illustrated for case 2 (figure 1). A diurnal periodicity of whole blood amino acids was apparent with levels at 8 P.M. consistently greater than those obtained at 8 A.M. on the same day. The mean daily increase was 16±3% (1 SE).

The clinical and amino acid responses of the 16 infected subjects as well as the shams have been summarized in table 1. Fourteen subjects had abrupt onset of illness within 96 hours of exposure. Illnesses were characterized by fever, malaise, headache, myalgia, photophobia, nasopharyngitis, cough, and retrosternal chest pain. Individuals became clinically well within 48 hours of therapeutic intervention. All subjects ex-

† These studies were conducted in conjunction with a program for development and testing of vaccines for and therapy of acute infections. The investigations were supervised by the Commission on Epidemiological Survey of the Armed Forces Epidemiological Board.

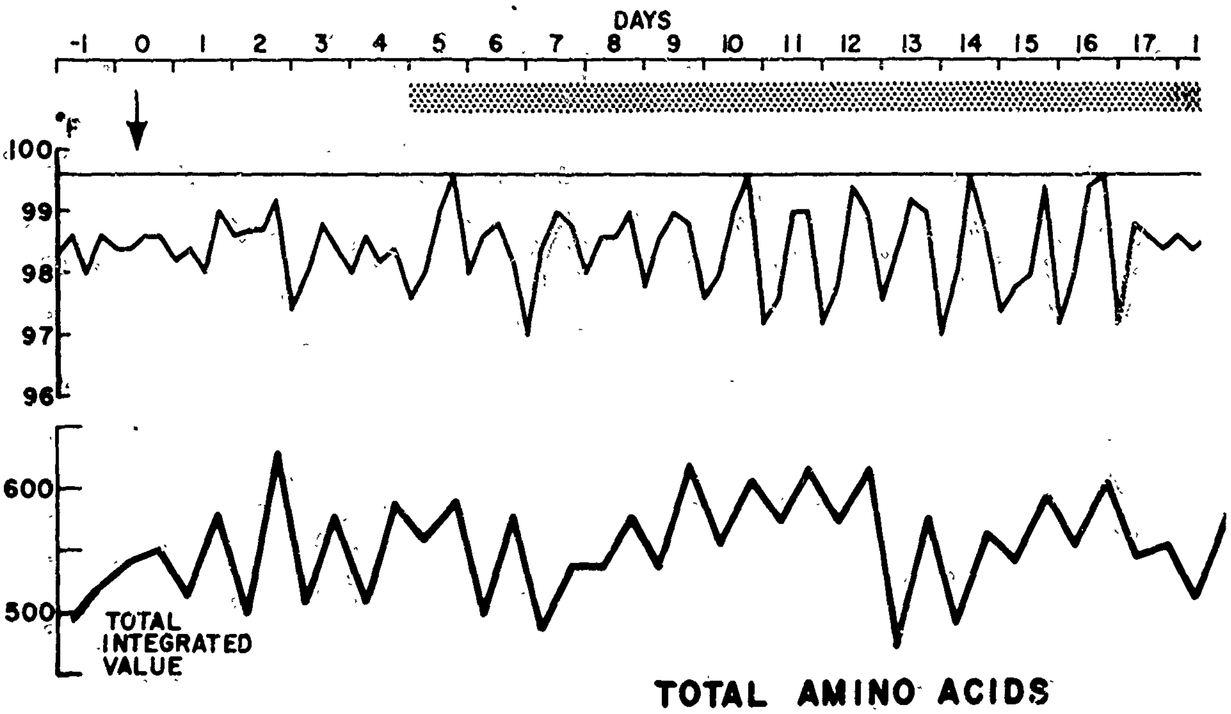

FIGURE 1.—Temperature and total amino acid responses of sham-exposed subject (case 2). Arrow indicates day of exposure. Stippled bar indicates tetracycline administration.

posed to *P. tularensis* developed positive bacterial agglutination titers, thus verifying infection, while sham-exposed subjects did not.

A decrease in total amino acids significant at $P<0.01$ when compared with the shams was observed in all 16 infected subjects within 12 to 48 hours after exposure. A maximal decrease was observed an average of 2.28 days after exposure. The mean ± 1 SE decrease was $43 \pm 4\%$ with a range of 18 to 63%. This decrease preceded the onset of symptoms in each instance. The decrease in the individually quantitated amino acids was found to be proportional to the decrease in the total integrated value. The extent of the decrease in amino acid concentration appeared to be unrelated to the severity of illness.

TABLE 1.—Clinical and amino acid responses in 18 subjects with respiratory-acquired tularemia

Case number	Amino acid decrease			Fever				Therapy,† day of onset	Amino acid increase			Illness index‡
	Day of onset	Day of maximum	Per cent	Day of onset	Peak degrees, F	Hours >99.6	Last day		Day of onset	Day of maximum	Per cent	
1*	1	1	9		0	0		5	9	11	20	0
2*	13	13	2		0	0		5	9	11	16	0
3	1	3	63	3	104.6	72	5	4	5	6	53	64
4	1	1, 4	28	3	102.6	48	5	5	4	4, 5	67	36
5	1	1	36	3	103.6	48	5	4	5	6	16	24
6	1	2	18	2	104.6	60	5	4	5	6	16	48
7	1	4	55	2	103.8	84	5	4	6	6	37	48
8	1	3	61	2	103.6	60	4	4	6	6	20	24
9	1	2	36	2	103.8	72	5	4	5	5	39	48
10	1	2	53	4	104.2	54	5	4	6	6	60	48
11	1	2, 3	39	3	103.8	48	5	5	6	6	18	24
12	1	1	36	3	102.6	48	5	4	6	6	5	27
13	1	1, 3	20	2	105.4	48	4	4	5	5	28	36
14	0§	1	33	3	104.2	36	5	5	0	0	0	16
15	1	2	56	3	103.2	46	5	5	4	5	25	32
16	2	3	50	3	103.6	48	4	5	0	0	0	24
17	1	1	44	3	See details in text		—	—	0	0	0	6
18	1	4	57	3	See details in text				0	0	0	12

* Sham-exposed.
† 14 days of tetracycline.
‡ See text.
§ Twelve hours.

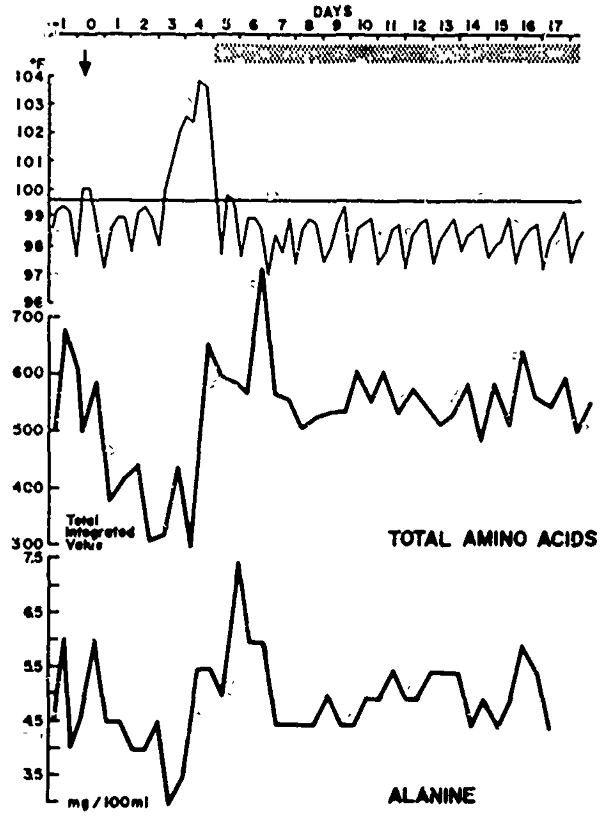

FIGURE 2.—Temperature, total amino acid, and alanine responses of infected subject (case 11). Arrow indicates day of exposure. Stippled bar indicates tetracycline therapy.

Within 6 to 72 hours following onset of fever 7 subjects developed an increase in amino acid concentration greater than that recorded at any time in the shams.

Typical responses of one of these subjects (case 11) are illustrated in figure 2. Total amino acids are plotted beneath rectal temperature. A marked fall in total amino acids is noted 48 hours prior to onset of fever. Initial clinical symptoms were noted on day 4. Lysis of fever and disappea ce of clinical symptomatology occu d within 24 hours of initiation of therapy. The result of quantitation of a single amino acid (alanine) is shown in the same figure to demonstrate that changes closely approximated those observed for the total.

The clinical and amino acid responses of 2 subjects (cases 17 and 18) were unusual. Fever, first noted on day 4 in both subjects, never exceeded 102 degrees (R) and was of brief duration. Subjective symptoms were mild in both cases. An initial decrease in total amino acids was noted 24 hours after exposure in each subject. Thereafter, concentrations varied greatly from day to day and apparently reflected the unusual clinical courses. Streptomycin therapy was begun on day 16.

DISCUSSION

Smith and Tempest (1957) demonstrated that anthrax in guinea pigs was accompanied by decreased quantities of glutamic acid, glycine, and threonine, while other amino acids remained constant or increased. Wakeman and Morrell (1930) described a terminal increase in blood amino acids in monkeys infected with yellow fever. Squibb (1966) has recently reported changes in the normally occurring circadian variation of amino acids in chickens infected with Newcastle disease virus. Finally, and most relevant to the studies being reported, Woodward et al (1954) noted a decrease in serum amino acids of white rats following 1 LD_{50} of *P. tularensis* (Sm strain). This change was recorded 72 hours after inoculation in all rats and in 4 of 12 as early as 48 hours.

The only reports of whole blood amino acid changes during infection in humans are those of Farr et al (1940) who observed a fall in plasma amino acid nitrogen during pneumococcal pneumonia and Emerson et al (1943) who noted that similar changes did not occur with primary atypical pneumonia.

The effects of tularemia on whole blood amino acids in man have not been reported previously. A pronounced fall in whole blood amino acids was observed 12 to 60 hours following aerosol exposure to *P. tularensis*. This decrease was proportional to the total integrated values of the individual amino acids detectable by the method described. Decreased amino acid concentration was apparent in all infected subjects prior to the onset of fever or any other clinical signs of illness. One of us (RDF) was unaware of the identity of sham-exposed controls but accurately predicted absence of infection from the amino acid determinations. Following the initial drop, whole blood amino acid responses in 2 patients differed from those observed in the other infected subjects and apparently reflected their unusual clinical courses.

The factors responsible for the marked decrease in amino acid concentration are difficult to elucidate. Woodward et al (1954) observed that cystine, an essential metabolite for *P. tularensis*, disappeared from the blood of rats infected with highly virulent strains of the organism. They also noted decreased concentrations but not disappearance of the other free amino acids. In contrast, killed or living avirulent organisms did not induce a similar change. They hypothesized that localization of this organism in liver and spleen may result in utilization of free amino acids directly from the blood as it filters through these organs. The generation time of *P. tularensis* under optimal conditions in vitro is approximately 1 hour (Gaspar et al, 1960). Beginning with 1 organism, a count of 8×10^6 would be obtained at 24 hours. The generation time as well as the organisms' rate of cystine utilization for growth in man has not been established. Whether the reduction in whole blood amino acids in individuals with tularemia can be explained by Woodward's hypothesis is speculative. However, our findings of a proportional rather than selective decrease ·· cystine suggest that other factors may be operative.

The work of Pinchot et al (1949) directed attention to adrenal involvement in rats with tularemia. Adrenal hypertrophy with resulting exhaustion of these glands was observed. Bondy (1949) noted that adrenalectomy lowered free amino acids in plasma, while Dubreuil and Timaris (1953) demonstrated a rise following administration of cortisone acetate. It has also been demonstrated that activity of D-amino acid oxidase in liver and kidneys of tularemia-infected rats was inhibited (Woodward and Mayhew, 1956). Since adrenocortical hormones have been found to control, in part, the blood level of amino acids as well as hepatic enzymes involved in their metabolism, Woodward and Mayhew (1956) postulated that "adrenal involvement" with subsequent exhaustion may be responsible for the diminished amino acid level seen in the rat.

Increased excretion of glucocorticoids during human tularemia infection occurred with the onset of fever and abated concomitantly with clinical improvement (Beisel, 1965). Furthermore, during infection adrenal overactivity was roughly proportional to the clinical severity of the disease process. Since the initial fall in whole blood amino acids in man preceded the period of adrenal overactivity and occurred even in subjects with less severe clinical responses, this decreased amino acid concentration could not be explained by adrenal exhaustion.

The catabolic effect of glucocorticoids on body protein is well known. Since adrenal stimulation accompanies tularemia infection in man, one should expect an elevation in whole blood amino acids rather than a decrease. Amino acid increases were seen following fever in our subjects with the more severe clinical illnesses, with the exception of case 6. This rise may be the result of a steroid catabolic effect.

Beisel (1965) has demonstrated that diminished urinary excretion of urea and alpha-amino nitrogen occurred in the period immediately prior to the onset of clinical illness of individuals with respiratory tularemia. Studies of tularemia in rats have demonstrated that increased urinary amino acid loss does not occur (Woodward et al, 1954). It is apparent that the changes noted in the blood are reflected in the urine and that lowered blood levels cannot be accounted for by urinary losses.

Significant increases in beta and gamma globulins as well as associated decreases in blood urea nitrogen, nonprotein nitrogen, and serum albumin levels have been described within 24 hours of *P. tularensis* infection in rats (Sbarra and Woodward, 1955; Woodward and Miraglia, 1964). It is attractive to speculate that *P. tularensis* in man has initiated similar metabolic changes resulting in the utilization of whole blood amino acids for new protein synthesis including antibody formation within hours of infection. Confirmation of this hypothesis requires additional studies.

Neither of the sham-exposed subjects showed increased or decreased concentrations of whole blood amino acids; tetracycline administration had no effect. The decreased amino acid concentrations preceded illness in all subjects. Therefore, it would appear that changes noted in infected subjects must be related to their infection. The specificity of these changes for tularemia can be determined only by additional studies of other human infections.

SUMMARY

Significant changes in the whole blood amino acids of volunteers with respiratory-acquired *Pasteurella tularensis* (SCHU-S4) infection have been described. A decrease in whole blood amino acids occurred 12 to 60 hours after exposure and prior to the time that clinical symptomatology was apparent. The factors responsible for this decreased amino acid concentration were discussed but remain undefined. These changes were specifically related to infection, in that sham-exposed and similarly treated subjects did not manifest a similar response. The pattern of response was such that, in retrospect, it appeared to have predictive value for determining which subjects had been infected.

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