Studies on the Blood Type Determination of Human Hairs by Ultrasonic Waves

Report 1. Elution test of hairs by ultrasonic waves

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In the practical field of legal medicine, the necessity arises frequently in connection with the identification of hairs, even a single hair, and fragments of tissues found at the scene of a crime or accident. Up to the present time, the personal identification of hairs has been done synthetically by morphological examinations,[1] quantitative analyses,[2)3)4)] determination of blood groups and so on.[5)6)7)] Among them, the determenation of blood groups is most important.

In 1929, KAYANO[8)] crushed hairs physically to extract blood group substances. SAKAI (1951),[5)] TSUTSUMI (1960)[9)] et al tried to extract blood group substances from hairs softened chemically with urea, karium salfide, ethanol etc. After that, hairs have been used for the determination of blood groups.

MURAKAMI (1964)[10)] and AKAISHI (1965)[11)] used the group specific double combination reaction to modify the mixed agglutination test in order to determine the blood groups of hairs. In 1966, the elution test was utilized for the determination of blood groups of hairs by YADA[12)] et al. Under these circumstances, methods for the determination of hairs have been improved.

A few years before YADA's experiments, KIND (1960)[13)14)] or NICKOLLS and PEREIRA (1962)[15)] first introduced a new technique called the elution test for the blood grouping of blood stains. The principle lies in that the agglutinins absorbed into the blood stains are eluted and the presence or absence of the agglutination of the correspondent indicator cells added to the eluates are examined for the blood grouping. A supplementary examination of this elution test was tried by OUTTERRIDGE (1962)[16)17)] and FIORI (1963).[18)] In Japan, YADA (1966)[19)] improved this elution test and applied it to the blood grouping of hairs.[12)] After that, many investigators studied the elution test and now the test has been widely used in the practical field of legal medicine.[20-23)] However, the determination of the blood grouping of hairs by means of an elution test presents

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difficulties, compared with that of blood stains and body fluid stains. Even for those who have been well trained, it is hard to use the elution test accurately for hairs. Hairs are one of human hard tissues and have less blood group substances than blood stains etc. So the absorptions of blood group antigens of hairs are very difficult.

Agglutinin absorption tests of blood stains, organs etc. were studied by using the irradiation of ultrasonic waves or sound waves at our institute and we succeed ed in obtaining good results.[24-28] So, in this present study, an attempt was made to utilize the irradiation of the ultrasonic waves in the elution test of hairs.

Ultrasonic waves have been utilized widely in the fields of chemical industries, medicine, biology etc.[29)31)] The actions or advantages are the accerelation of chemical reactions, destruction of tissues, washing and release of specific elements etc.[29)31)] These actions, especially the destructive action, may depend on the cavitation and vibration of ultrasonic waves.[29)] From the viewpoint mentioned above, we studied various methods for utilizing ultrasonic waves for the agglutinin absorption of the elution test in the determination of ABO blood type of hairs.

MATERIALS

As an ultrasonic generator, the multiple wave generator (T-A-4014 Type) made by KAIJO DENKI Co. was used and the exposed strength was fixed at 40 w. Head hairs used as specimens were obtained from humans whose blood groups were already known to us. They were washed with neutral cleanser, then rinsed with ether free from any fatty substances and finally dried. The antisera employed for the present experiment was made from natural blood serum which was freshly prepared prior to each test. The blood serum was incubated at 56°C for 30 minutes to inactivate it. Then sodium fluoride was added to it in a ratio of one to one thousand. These were stored in an ice box. The titers of both anti-A and anti-B used were 128-256. A 0.2 % suspension of indicator cells was used.

METHODS

Hairs, approximately 4cm long, injured mechanically and chemically were diveded into two groups. Each of them was placed in a test tube. Three drops of anti-A reagent were added to one and the same number of drops of anti-B reagent to the other. Then the experimental groups were irradiated with various degrees of ultrasonic waves. On the other hand, the control groups were incubated at room temperature for 3 hours without the irradiation of ultrasonic waves. After that, the supernatant antisera were carefully pipetted off and the hairs were washed twice with cold saline to be free of uncombined agglutinins. After the addition of three drops of saline, the mixtures were again incubated in a water bath at 50-55°C for

ten minutes to eluate the absorbed antisera. One drop of group A or group B indicator cells was added to the eluates and the mixtures were gently agitated. After 30 minutes, they were centrifuged at 1000 r.p.m. for one minute and were examined for the presence or absence of the agglutination with the aid of a concave mirror. The absence of the agglutination was shown with the sign-, and in proportion to the strength of an agglutination, the signs were shown as $^{+}$, $^{++}$, or $^{+++}$.

RESULTS

I Preliminary treatment of hairs

Hairs, which were washed, freed from any fatty substances and finally dried, were crushed, swollen, heated, or decolorized. The elution test was performed on each set of hairs and the results were compared with that of the control experiment with untreated samples.

1. Crushed Method

Hairs, about 4cm long, wrapped in a sheet of thin paper, were uniformly crushed with the use of a small hammer. The injured specimens were then cut into short segments, about 0.5cm in length and divided into two groups. Each of them was placed in a test tube and the elution test was performed.

2. Swollen Method

The swollen method consisted of placing hairs, about 4cm long, at 10cm above a small flame of an alcohol lamp to cause slight swelling without causing charing. After that, the elution test was performed as mentioned above.

3. Heated Method

Hairs, about 4cm long, were placed in an evaporating dish and the dish was removed to a dry sterilization chamber at 100 or 200°C for one hour. Then the elution test was performed.

4. Decolorized Method

Hairs, about 4cm long, were decolorized with a 3 % hydrogen peroxide solution for 3 days. The elution test was performed with the use of these hairs.

As the control experiment, hairs, about 4cm long, which were washed, freed from any fatty substances and finaly dried, were used without any other treatments.

The results obtained (see Table 1) were as follows : The crushed method was the best, making a definite judgement of the type possible in all samples. The swollen method was slightly inferior, giving rise to reactions about one half weaker than the crushed method in samples of each type except for type B. The result of the type B was equal to that of the crushed method. However, the judgement was possible in each type.

In the heated method and the decolorized method, the judgement of the type was difficult except that of type B, which could be accurately judged.

Table 1. Preliminary treatments of hairs

blood types of samples \ preliminary treatments: anti serum	crushed method	swollen method	heated method 100°C	heated method 200°C	decolorized method	untreatd control
A	anti-A	+	±	−	−	−	−
	anti-B	−	−	−	−	−	−
B	anti-A	−	−	−	−	−	−
	anti-B	++	++	±	++	+	−
AB	anti-A	±	±	−	±	−	−
	anti-B	+	+	−	±	+	−
O	anti-A	−	−	−	−	−	−
	anti-B	−	−	−	−	−	−

In the control experiment with untreated samples, the judgement of each type was impossible.

II Utilizations of ultrasonie waves

As presented in part I, the crushed method and the swollen method were good for preliminary treatments of hairs. These two methods were used to utilize the irradiation of ultrasonic waves in the procedure of antiserum absorption of hairs.

1. Optimal cycles in the utilization of ultrasonic waves.

Using each of 20, 50, 100, 200, 400 and 600 KC, 1.2 and 1.5 MC, ultrasonic wave irradiations were given for 30 minutes. As presnted in Tables 2 and 3, at each cycle between 20 and 600 KC, the effects of the irradiation were similar. The result was about one step superior to that of the non-irradiated control group in all samples, while any differences from the non-irradiated control group were not seen at 1.2 and 1.5 MC, which produced no observable effects of irradiations. The intensity of the reaction was about one step superior with the use of the crushed method than with the use of the swollen method.

Table 2. Optimal cycles in the ultrasonic wave irradiations for 30 minutes with the use of the crushed method

blood types of samples \ cycles: anti serum	20 (KC)	50	100	200	400	600	1.2 (MC)	1.5	non-irradiated control
A	anti-A	++	++	++	++	++	++	+	+	+
	anti-B	−	−	−	−	−	−	−	−	−
B	anti-A	−	−	−	−	−	−	−	−	−
	anti-B	++	++	++	++	++	++	++	++	++
AB	anti-A	+	+	+	+	+	+	±	±	±
	anti-B	++	++	++	++	++	++	+	+	+
O	anti-A	−	−	−	−	−	−	−	−	−
	anti-B	−	−	−	−	−	−	−	−	−

Table 3. Optimal cycles in the ultrasonic wave irradiations for 30 minutes with the use of the swollen method

blood types of samples \ cycles / anti serum		20 (KC)	50	100	200	400	600	1.2 (MC)	1.5	non-irradiated control
A	anti-A	+	+	+	+	+	+	±	±	±
	anti-B	−	−	−	−	−	−	−	−	−
B	anti-A	−	−	−	−	−	−	−	−	−
	anti-B	##	##	##	##	##	##	##	##	##
AB	anti-A	+	+	+	+	+	+	±	±	±
	anti-B	##	##	##	##	##	##	+	+	+
O	anti-A	−	−	−	−	−	−	−	−	−
	anti-B	−	−	−	−	−	−	−	−	−

2. Adequate durations of irradiations in utilizing ultrasonic waves.

Irradiations were conducted for 5, 15, 30, 60, 90 and 120 minutes and the cycle was limited to 20, 200 and 600 KC based on the experimental results described above. The optimal duration of the irradiations and the relationship with the cycle were comparatively studied. The results obtained (seeTables 4 to 9) were as follows: As for the duration of the irradiations, 5 minutes was definitely insufficient for the use of the crushed method, and 15 minutes was still somewhat deficient. The results from the 15 minute irradiations were about one half to one step inferior to the non-irradiated control group, while any differences from the non-irradiated control group were not seen at 20 KC. but the results of type B were about one step superior.

Thirty minutes was sufficient and the type specific reaction was about one step superior to that of the non-irradiated control group.

Sixty minutes was sufficient, the same as 30 minutes. Ninety and 120 minutes were, in general, as adequate as 30 and 60 minutes but were a little too long at 20 KC, giving rise to a type non-specific reaction. One hundred and twenty minutes was also too long at 200 KC. In general, a long irradiation at a low cycle was found to be harmful. Such a tendency was also similarly seen with the use of the swollen method.

III Preliminary treatments influencing the absorption of antibodies by hairs and the relationship with ultrasonic wave irradiations.

Hairs subjected to the crushed, swollen, heated or decolorized methods, as mentioned above, were irradiated with 200 KC as the optimal cycle of ultrasonic waves with 30 minutes as the adequate duration. The intensity of antibody absorption was compared by method. The results obtained were as follows (see Table 10): The crushed method was definitely the best among preliminary treatments of hairs with the use of ultrasonic waves. The resuts were about one step superior to that

of non-irradiated controls of the crushed method.

The swollen method was the second. The results were about one half to one step better than the corresponding non-irradiated controls.

The decolorized method was the third and gave rise to reactions about one half to one step better than the corresponding non-irradiated controls.

The fourth was the heated method. The results at both 100 and 200°C were a half step better than that of non-irradiated controls. And some untreated irradiated controls gave rise to reactions about a half step superior to untreated non-irradiated controls (see Tables 1 and 10).

However, there was no difference between irradiated hairs and non-irradiated ones in the way of superior results in the type specific reactions.

The crushed method gave a better outcome than the other methods of prelimina ry treatments in the irradiated samples and also non-irradiated ones.

The blood type determination of hairs by the use of the elution test was best in hairs which were prepared by the crushed method and irradiated for a half an hour during the antibody absorption period.

Table 4. Optimal durations in the ultrasonic wave irradiations at 20 KC with the use of the crushed method

blood types of samples \ irradiation time / anti serum	5 (minutes)	15	30	60	90	120	non-irradiated control
A	anti-A	−	+	++	++	++	++	+
	anti-B	−	−	−	−	±	±	−
B	anti-A	−	−	−	−	−	−	−
	anti-B	+	+++	+++	+++	+++	+++	++
AB	anti-A	−	±	+	+	+	+	±
	anti-B	+	+	++	++	++	++	+
O	anti-A	−	−	−	−	±	±	−
	anti-B	−	−	−	−	±	±	−

Table 5. Optimal durations in the ultrasonic wave irradiations at 200 KC with the use of the crushed method

blood types of samples \ irradiaton time / anti serum	5 (minutes)	15	30	60	90	120	non-irradiated control
A	anti-A	−	+	++	++	++	++	+
	anti-B	−	−	−	−	−	±	−
B	anti-A	−	−	−	−	−	−	−
	anti-B	+	++	+++	+++	+++	+++	++
AB	anti-A	−	±	+	+	+	+	±
	anti-B	±	+	++	++	++	++	+
O	anti-A	−	−	−	−	−	±	−
	anti-B	−	−	−	−	−	±	−

Table 6. Optimal durations in the ultrasonic wave irradiations at 600 KC with the use of the crushed method

blood types of samples \ irradiation time / anti serum	5 (minutes)	15	30	60	90	120	non-irradiated control
A	anti-A	−	+	++	++	++	++	+
	anti-B	−	−	−	−	−	−	−
B	anti-A	−	−	−	−	−	−	−
	anti-B	+	++	+++	+++	+++	+++	++
A B	anti-A	−	−	+	+	+	+	±
	anti-B	−	±	++	++	++	++	+
O	anti-A	−	−	−	−	−	−	−
	anti-B	−	−	−	−	−	−	−

Table 7. Optimal durations in the ultrasonic wave irradiations at 20 KC with the use of the swollen method

blood types of samples \ irradiation time / anti serum	5 (minutes)	15	30	60	90	120	non-irradiated control
A	anit-A	−	±	+	+	+	+	±
	anti-B	−	−	−	−	−	±	−
B	anti-A	−	−	−	−	−	−	−
	anti-B	+	++	+++	+++	+++	+++	++
A B	anti-A	−	−	+	+	+	+	±
	anti-B	±	±	++	++	++	+++	+
O	anti-A	−	−	−	−	−	−	−
	anti-B	−	−	−	−	−	−	−

Table 8. Optimal durations in the ultrasonic wave irradiations at 200 KC with the use of the swollen method

blood types of samples \ irradiation time / anti serum	5 (minutes)	15	30	60	90	120	non-irradiated control
A	anti-A	−	±	+	+	+	+	±
	anti-B	−	−	−	−	−	−	−
B	anti-A	−	−	−	−	−	−	−
	anti-B	+	++	+++	+++	+++	+++	++
A B	anti-A	−	−	+	+	+	+	±
	anti-B	−	±	++	++	++	++	+
O	anti-A	−	−	−	−	−	−	−
	anti-B	−	−	−	−	−	−	−

Table 9. Optimal durations in the ultrasonic wave irradiations at 600 KC with the use of the swollen method

blood types of samples \ irradiation time / anti serum	5	15	30	60	90	120	non-irradiated control
A	anti-A	−	−	+	+	+	+	±
	anti-B	−	−	−	−	−	−	−
B	anti-A	−	−	−	−	−	−	−
	anti-B	−	+	++	++	++	++	++
AB	anti-A	−	−	+	+	+	+	±
	anti-B	−	−	++	++	++	++	+
O	anti-A	−	−	−	−	−	−	−
	anti-B	−	−	−	−	−	−	−

Table 10. Ultrasonic wave irradiation at 200 KC for 30 minutes after preliminary treatments

blood types of samples \ preliminary treatments / anti serum	crushed method	swollen method	heated method 100°C	heated method 200°C	decolorized method	untreated control
A	anti-A	++	+	−	−	±	−
	anti-B	−	−	−	−	−	−
B	anti-A	−	−	−	−	−	−
	anti-B	++	++	+	++	++	±
AB	anti-A	+	+	−	±	−	−
	anti-B	++	++	±	+	±	±
O	anti-A	−	−	−	−	−	−
	anti-B	−	−	−	−	−	−

DISCUSSION

Since the authors[24-27] have already experienced positive effects of ultrasonic irradiations in the defermination of blood types through the agglutinin absorption test, this was applied to the elution test for hairs in the present study.

(1) Preliminary treatments of hairs

The method of conducting absorption and elution through adding antiserum directly to washed, defatted and dried hairs was used as the untreated control groups, while hairs which were crushed, swollen, heated, or decolorized weve used as treated groups for comparison. The results in the untreated groups were inferior to those in the treated groups. Except for some samples of type B, the determination of blood type was not possible in almost all untreated cases. In the treated groups, the crushed method was the best, making a definite determination possible in samples of each type. The swollen method was slightly inferior, giving

rise to reactions about one half weaker than the crushed method in samples of each type except for type B. However, a judgement was possible in each type. On the contrary, in the heated method and the decolorized method, the determination of the type was somewhat more difficult. In the examination of blood type using hair samples, various methods of treatment have been devised in the early 20th century physically,[8)12)] chemically[5)9)] or through their combination. In order to conduct the elution test of hairs, the crushed method[23)] appears to be used most widely at present. Before utilizing ultrasonic irradiations, various preliminary treatments such as the crushed, swollen, heated, and decolorized methods were comparatively investigated. The results showed that the crushed method gave a better outcome than the other three. This is probably due to the fact that the procedure of the crushed method simply dilates the surface area of the hair without causing the degeneration of blood group antigen substances in the hairs. The surface is also made rough and softened to facilitate the antigen-antibody reaction.

(2) Optimal cycles in the utilization of ultrasonic waves

Using each of 20, 50, 100, 200, 400 and 600 KC, 1.2 and 1.5 MC, ultrasonic wave irradiation was given for 30 minutes. The crushed method and the swollen method were used to compare the experimental results at each of these cycles. As the result, at each cycle between 20 and 600 KC, the effect of irradiation was similar. The result was about one step superior to the non-irradiated control group in all samples, while scarcely any difference from the non-irradiated control groups was seen at 1.2 and 1.5 MC. At these levels no effect of irradiation was seen in both the crushed method and the swollen method. The intensity of the reaction was about one step superior with the use of the crushed method to the use of swollen method. It is said that EULER (1956)[29)] was the first to note the phenomenon of cavitation. This is the phenomenon of the development of an air pocket within the fluid due to decompression, caused by static negative pressure, dynamic strength, sound waves etc. That which occurs in response to irradiations of intense ultrasonic waves is called ultrasonic cavitation.[29)31)] On the other hand, as the cycle of the sound becomes higher, cavitation starts to decline, as reported by GAERTNER.[30)]

According to our experimental results obtained during the study in search of the optimal cycle, favorable results were obtained at a relatively low cycle range of 20 to 600 KC. This fact might be explained by the characteristics of ultrasonic waves, producing more intense cavitation as the cycle is lower. In the low cycle of 20 to 50 KC, intense cavitation and vibration were seen to cause the jumping of test hairs out of the antiserum. This phenomenon apparently contradicts the purpose of making the absorption of the antibody by the hair complete. Then, the hair was sunk into the antiserum occasionally using glass rods. The cavitation gradually diminished between 100 and 600 KC, and the hair scarcely jumped between 200 and 600 KC. Based on these observations, 200 or 600 KC appears

to be adequate for the actual ultrasonic irradiation.

(3) Adequate durations of irradiations in utilizing ultrasonic waves

Irradiation was conducted for 5, 15 30, 60, 90 and 120 minutes and the cycle was limited to 20, 200 and 600 KC based on the experimental results described above. Hairs were treated by the crushed or swollen method, to study comparatively the optimal duration of irradiation and the relationship with the cycle. As for the duration of irradiation, 5 minutes was definitely insufficient with the use of the crushed method, 15 minutes was still somewhat deficient, 30 or 60 minutes appeared to be adequate, and 90 or 120 minutes was apparently a little too long, according to the results obtained. Such a tendency was also similarly seen with the use of swollen method. The intensity of the reaction, as in the study on the cycle number described above, was slightly more with the use of the crushed method than with the use of the swollen method. It appears possible therefore to shorten the time of absorption from 3[23] hours to 15 or 30 mnutes upon utilizing ultrasonic wave irradiations in the procedure of antibody absorption of the hair. The time is shortened as the cycle becomes lower, and a long term irradiation was found to be harmful. These phenomena are apparently to a rapid termination of the reaction on account of the powerful cavitation and vibration induced by ultrasonic waves facilitating the absorption of the antibody into hard tissue and, in addition, raising the frequency of contact between antigen and antibody. Consequently, the fact that a long term irradiation at a low cycle gives rise to a type non-specific reaction might be explained by the release or extraction of excessive antibodies through sustained intense ultrasonic action even after the completion of reaction within a short time. Based on these facts, the adequate duration of irradiations appears to be as short as possible, 30 minutes, for the original purpose of the test.

(4) Preliminary treatments influencing the absorption of antibodies by hairs and the relationship with ultrasonic wave irradiations

Usually the crushed method is considered the best among preliminary treatments of hairs.[12][20][23] The heated and decolorized methods are not utilized. However, all these methods were studied for the purpose of elucidating the relationship between the physical action of ultrasonic waves and the method of the preliminary treatment of hairs. In the experiment I described above, the elution test without ultrasonic irradiations was conducted on hairs subjected to the various preliminary treatments described above. In experiment II, the most advantageous cycle and the time of irradiations utilizing ultrasonic waves were studied. Furthermore, the introduction of ultrasonic waves was attempted in the elution test of hairs subjected to various preliminary treatments. Hairs subjected to crushing, swelling, heating, and decolorizing were irradiated with 200 KC ultrasonic waves for 30 minutes along with the untreated controls, to compare the experimental results. The results were most favorable in the crushed method followed by the swollen

method and then the decolorized method. The results, which made the type determination possible, were one-half to one step better than the corresponding non-irradiated controls. With the heated samples and all untreated samples, the effects of irradiations were partially seen, giving a difference of about a half step, and the type determination was not possible.

In the crushed method, the manipulation of crushing softens the hairs and makes the surface of the hairs rough, to enlarge the surface area raising the frequency of the contact between the antigen and the antibody or between reactive elements to facilitate and accelerate the reaction. Ultrasonic wave irradiations probably accentuate such an effect.

As for the physical action of the ultrasonic waves, destruction of the tissues, release or extraction of specific elements following such destructions, and mixing of particles or in a molecular state of such substances probably take place. It is said that such a mechanical action can combine even molecular opposites to each other.[29] When the antigen antibody reaction is considered from the immunological viewpoint, such mechanical actions might possibly cause pseudoreaction. In the present experimental results, type non-specific reactions seen upon long term irradiations with low cycle waves might belong to this category. Under the condition of the optimal irradiation, such considerations were not necessary. As stated in the experimental result I-2, the swollen method consisted of placing the hair above a small flame to cause slight swelling and curling without causing charing. Under the microscope, the hair was swollen forming a large number of cavities. The surface was relatively smooth. Therefore this method was used to prevent the pseudoreaction described above. As a result, the pseudoreaction was somewhat prevented as compared with the crushed method but the reaction was generally weakened. This does not appear to be an optimal method. According to the present results, however, the swollen method may possibly be used for rhe test of the blood grouping of hairs in the mixed agglutination test. Concerning the heat degeneration of blood group substances, heating at 240°C for one hour already destroys the blood group substances according to Watanabe.[21] Brown discoloration, curling, and weakening of the hairs occurs at 190 to 250°C. In the present experiment, the lowest limit of temperature causing swelling and curling appears to be about 250°C, according to the measurement with a thermometer placed over a small flame along with the hair. At this temperature, swelling and curling occurs only through momentary and unbalanced heating through a flame. When homogenous heating with a dry sterilizer is used, no swelling and curling occurs even upon heating at 250°C. Definite judgements of the type are possible even at such a dangerously critical temperature probably because the heating was instantaneous. The heated method at both 100 and 200°C and the decolorized method were unsuitable for the preliminary treatment of hairs in irradiated or non-irradiated groups.

Based on these observations for the blood type determination of hairs by the use of the elution test in irradiated or non-irradiated groups, the preliminary treatment such as the crushed method, etc. is necessary to soften hard tissues.

In all samples, the differences in intensity were noted between reactions of anti-A and anti-B serum. In spite of using anti-A serum on equal titers of the antibody with anti-B serum, the reactions of type A were inferior than those of type B. Such a result was already obtained by KITAHAMA[23] et al. and they suggested these were due to a general characteristic in anti-A serum.

As described above, the elution test by the use of ultrasonic wave irradiations appears to be sufficiently useful in the place of the conventional non-irradiated test, with many advantages such as shortening of the time of absorption and the clarification of type judgements.

CONCLUSION

The elution test by the use of ultrasonic wave irradiations appears to be sufficiently useful for the blood type determination of hairs in the place of the conventional non-irradiated test.

The advantages were shortening of the absorption time and clarification of blood types.

The optimal cycle of ultrasonic waves during the antibody absorption period was 200 KC and the optimal duration was 30 minutes.

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