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

Report 2. Mixed Agglutination Test of Hairs by Ultrasonic Waves

Setsuo KOMURA and Sakuko HAYASHI
Department of Legal Medicine,
Yamaguchi University School of Medicine
(Director : Prof. Junji Furuno)
(Received November 2, 1970)

The elution test by the use of ultrasonic wave irradiations appeared to be sufficiently useful for the blood type determination of hairs, as shown in report I,[1] previously reported. The advantages were shortening of the absorption time and clarification of blood types. Coombs and Bedford (1955)[2] obtained platelets separately and sensitized them with type-specific serum. They demonstrated an agglutination reaction between these sensitized platelets and erythrocytes by adding corresponding red cells. This they called the mixed erythrocyte-platelet agglutination. Coombs and Dodd (1961)[3] tried to test for the presence or absence of type specific antigenic substances in a blood stain through the combination of blood cells in that blood stain with antiserum after the absorption of antiserum to the blood stain. They established a new method of blood type determination, the mixed agglutination test. Since this discovery, this test has been utilized as the method of blood type determination which requires only a minute amount of the sample and as a simple good method for the determination of blood types not only in blood stains[3-5] but also in other body fluid stains,[6] tissue cells,[2,6-9] hairs[10-12] and nails.[10,11] As reported previously, various methods of testing the blood types of hairs have been introduced and studied by many investigators. The mixed agglutination test had been applied for this purpose as a predecessor of the elution test in current use.[10-12] However, the technic for the test is so difficult that even a skillful worker finds it hard to obtain satisfactory results constantly. These disadvantages should be studied further.

In this present report ultrasonic wave irradiations were applied to the mixed agglutination test which was similar to the elution test. The authors studied the mixed agglutination test by the use of ultrasonic waves and obtained some results for preliminary treatments of hairs or optimal conditions of ultrasonic waves.

Portions of this report were presented at the 34th Scientific Congress of the Yamaguchi University Medical Association, Ube, 1969 and the 53rd Conference of the Medico-Legal Society of Japan, Hiroshima, 1969.

MATERIALS

The ultrasonic generator, head hairs used as specimens, the antisera and the indicator cells were the same as reported previously as report 1.

METHODS

Hairs, approximately 2 cm long, preliminary treated were divided into two groups. The specimens were then cut into short segments, 0.5 cm in length. Each of them was placed in a test tube. Three drops of anti-A regent were added to one and the same number of drops of anti-B regent 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 pipetted off and the hairs were washed twice with cold saline to be free of uncombined agglutinins. One drop of group A or group B indicator cells was added to the hairs which were placed on the hole glass. After the mixtures were incubated at room temperature for an hour, they were examined microscopically for the presence or absence of the agglutination to the hair. The presence of the agglutination to the whole hair was shown with the sign ##, the absence of the agglutination to the hair with the sign −, and in proportion to the strength of an agglutination, the signs were shown as ±, +, #, ##, or ##.

RESULTS

I. Preliminary treatment

Hairs were crushed, swollen or decolorized for the preliminary treatment. The mixed agglutination 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 2 cm long were crushed as same as reported previously. The injured specimens were cut into short segments, about 0.5 cm in length and divided into two groups. Each of them was placed in a test tube and the mixed agglutination test was performed.

2. Swollen Method

Hairs, about 2 cm long were swollen as same as reported previously. Then, the mixed agglutination test was performed as mentioned above.

3. Decolorized Method

Hairs, about 2 cm long were decolorized as same as reported previously. Then, the mixed agglutination test was performed.

4. Control Experiment

Hairs, about 2 cm long, which were washed, freed from any fatty substances and finally dried, were used without any other treatments. After that, the mixed agglutination test was performed.

The results obtained (see Table 1) were as follows: The swollen method gave rise to the type specific reaction in each type, but the reaction was slightly weak. The judgement was possible with difficulty. In the crushed method, the reaction was generally strong but the type non-specific reaction occurred. The type determination was somewhat indistinct. In the decolorized method, the determination of blood type was not possible in all cases. In the control experiment with untreated samples, the determination of each type was impossible.

Table 1. Preliminary treatments of hairs

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

II. Utilizations of ultrasonic waves

As presented in part I, the swollen method was the best and the crushed method followed 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 same as reported previously report 1. In the swollen method, as shown in Table 2, at each cycle between 200 and 600 Kc, the agglutinations were about one step superior to that of the non-irradiated control group. The type determinations were definite. At each cycle between 20 and 100 Kc, the results were about one to one and a half step superior to that of the non-irradiated control group, while the type determinations were indistinct in each type, giving rise to a type non-specific reaction. At each cycle both 1.2

Table 2. 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	+	+	±	−	−	−	−	−	−

Table 3. 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	++	++	++	+	+	+	±	±	±

and 1.5 Mc, the results were only about a half step superior to that of the non-irradiated control group. In the crushed method, as shown in Table 3, at each cycle between 20 and 600 Kc, the agglutinations were superior to that of the non-irradiated control group, but the type non-specific reaction occurred, especially at a low cycle. The type determination was difficult. Each cycle both 1.2 and 1.5 Mc slightly gave rise to a type non-specific reaction in the blood type A and O but the type determination was possible.

2. Optimal durations of irradiations in utilizing ultrasonic waves

Irradiations were conducted for 5, 15, 30, 60 and 90 minutes and the cycle

was limited to 200, 600 Kc and 1.5 Mc bases on the experimental results described above. The optimal duration of the irradiations and the relationship with the cycle were comparatively studied.

The results obtained for the use of the swollen method (see Tables 4 to 6) were as follows: As for the duration of the irradiations, 5 minutes was insufficient and at each cycle both 200 and 600 Kc the type B determination only was possible. The results from 15 minute irradiations were about one step superior to the non-irradiated control group at both 200 and 600 Kc. The type determination was possible. The results from 15 minute irradiations in the blood type AB at 1.5 Mc were about a half step inferior to that of the non-irradiated control group.

Table 4. Optimal durations in the ultrasonic wave irradiations at 200 Kc with the use of the swollen method

blood types of samples \ irradiatoin time / anti serum	5 (minutes)	15	30	60	90	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 600 Kc with the use of the swollen method

blood types of samples \ irradiation time / anti serum	5 (minutes)	15	30	60	90	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 1.5 Mc with the use of the swollen method

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

Thirty minutes was sufficient at both 200 and 600 Kc as same as 15 minutes. At 1.5 Mc the type specific reaction was about a half step superior to that of the non-irradiated control group.

Sixty minutes was sufficient at 600 Kc and 1.5 Mc but was a little too long at 200 Kc, giving rise to the type non-specific reaction.

Ninty minute irradiations were a little too long at both 200 and 600 Kc, giving rise to the type non-specific reaction but the irradiations at 1.5 Mc were sufficient as same as the 30 minute irradiations.

The results obtained for the use of the crushed method (see Tables 7 to 9) were as follows : In 5 minute irradiations the type B determination only was possible with difficulty at all cycles but the other type determinations were impossible because of the deficiencies of irradiation time. In 15 minute irradiations, the agglutinations were superior to that of 5 minute irradiations at each of 200 and 600 Kc and 1.5 Mc in the all blood types. The type determination was slightly difficult, giving rise to the type non-specific reaction. Thirty minute irradiations gave rise to the type non-specific reaction at both 200 and 600 Kc and the type determination was indefinite. At 1.5 Mc the results from 30 minute irradiations were about a half step superior to that of the corresponding non-irradiated control group and the type determination was possible. The results from 60 or 90 minute irradiations were as same as that of 30 minute irradiations.

Table 7. Optimal durations in the ultrasonic wave irradiations at 200 Kc with the use of the crushed method

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

Table 8. 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	non-irradiated control
A	anti-A	±	++	++	++	++	+
	anti-B	−	±	+	+	+	±
B	anti-A	−	±	+	+	+	±
	anti-B	+	+++	+++	+++	+++	++
AB	anti-A	−	++	++	++	++	+
	anti-B	+	++	+++	+++	+++	++
O	anti-A	−	±	+	+	+	±
	anti-B	−	±	+	+	+	±

Table 9. Optimal durations in the ultrasonic wave irradiations at 1.5 Mc with the use of the crushed method

blood types of samples \ irradiation time / anti serum	5 (minutes)	15	30	60	90	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 irradiations at 200 Kc for 15 minutes after preliminary treatments

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

III. Relationship between preliminary treatments and ultrasonic wave irradiations having influence on the absorption of antibodies by hairs

Hairs subjected to the swollen crushed or decolorized methods, as mentioned above, were irradiated with 200 Kc as the optical cycle of ultrasonic waves and 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 swollen method was definitly the best among preliminary treatments of hairs with the use of ultrasonic waves. The results in the swollen method were about one and a half to two step superior to that of the untreated controls. The type determination was most definite. The crushed method was the second. The results were about one and a half to two better to the corresponding untreated controls but a type non-specific reaction appeared slightly. The type determination was slightly inferior to that of the swollen methods. The third was the decolorized method which did not give rise to the type specific reaction for all blood types. The type determination was impossible.

DISCUSSION

1. Preliminary treatments of hairs

In the treated groups the crushed method was the best in the determination of blood types through the elution test, as reported previously. The second was the swollen method. These two methods and the decolorized method were used as treated groups for comparison. In the swollen method the determination of blood type was somewhat difficult but possible in samples of each type. The reactions

to anti-serum A in the type A or AB were inferior to that of anti-serum B and only a half step superior to the negative reaction. In the crushed method the reactions were somewhat stronger than that of the swollen method but a type non-specific reaction appeared slightly. The type determination was slightly indistinct. In the decolorized method all the reactions were same and weak in samples of each type. The type determination was impossible. In the control experiment with untreated samples all the reactions were negative and the determination of each type was impossible. As for the preliminary treatment of hairs in the elution test, the crushed method was recommended as the best, followed by the swollen method.

In the present mixed agglutination test, the swollen method appears to be the best one, followed by the crushed method. Preliminary treatments here represent the means of facilitating antibody absorption to hairs, through softening of the hairs and hard tissue by crushing and heating, as already, reported in a previous paper. However, the phenomenon of non-specific absorption appears only upon excessive destruction of hard tissue, as was pointed out by Yasuda et al.[13] in their studies on the mechanical destruction of hairs and absorption tests. This is readily surmised from the experience of the authors on non-specific absorption after excessive ultrasonic wave irradiations. Absorptions in the natural form are possible and appear to be most desirable. Especially in the mixed agglutination reaction, the blood type determination is made through the absence or presence of adhesion of blood cells on the surface of the hair. It is therefore mandatary to facilitate absorptions without damaging the surface of the hair. Our selection of the special swollen method in the previous report as the method of choice in the preliminary treatment was based on such a viewpoint. Although this made it possible to escape the phenomenon of non-specific absorption, it weakened the phenomenon of specific absorption.

In the mixed agglutination, a tendency towards non-specific adhesion of blood cells on the surface of the hair or secondary combination between the cells and the hairs was present besides the non-specific absorption reaction. The possibility of non-specific reaction in the mixed agglutination tended to be more pronounced than in elution test. Emphasis should therefore be placed on the inhibition of non-specific reactions rather than on the intensity of the reaction.

The swollen method appeared to be a good one as readily surmised from the previous report.

In the present experiment, the results were as expected.

The fact that weak non-specific reactions were seen in almost all cases after use of the decolorized method for the purpose of maintaining the natural form might indicate a simple non-specific adhesion of blood cells instead of an antigen-antibody reaction. This probably indicated the invasion of the tissue by hydrogen peroxide, causing a rough surface.

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 irradiations were given for 30 minutes. The swollen method and the crushed method were chosen for preliminary treatments of hair. The results were compared each other.

In the swollen method, at each cycle between 200 and 600 Kc, the agglutinations were more intensive than that of the non-irradiated control group. The type determination was definite. Each cycle between 20 and 100 Kc slightly gave rise to a type non-specific reaction and the type determination was indistinct. At each cycle between 1.2 and 1.5 Kc, the reactions were weak in each type and the irradiations were almost ineffective.

In the crushed method, at high cycle range of 1.2 to 1.5 Mc, the reactions were superior to that of the corresponding non-irradiated controls in each type but the type non-specific reaction occurred slightly in the blood type A and O. The type determination was comparatively definit. At each cycle between 200 and 600 Kc the agglutinations were stronger than between 1.2 and 1.5 Mc and the type determination was indistinct in order to give rise to a type non-specific reaction. Such a tendency was seen strongly at each cycle between 20 and 100 Kc and the type determination was almost impossible. A cavitation is called the basic phenomenon of intense ultrasonic waves within the fluid. The main actions of ultrasonic cavitation are the destruction of tissues and mixing of particles or in a molecular state of such substances.[14] As the cycle of the sound becomes lower, the cavitation starts to increase and the destructive action becomes stronger, as shown in report 1, previously reported. Both a type non-specific reaction at a low cycle and a decreased reaction at a high cycle appear to be due to such a distinctive character of ultrasonic wave. From the viewpoint mentioned above, the optimal cycles appeared to be between 200 and 600 Kc in the swollen method and between 1.2 and 1.5 Mc in the crushed method. At this time the ultrasonic wave irradiation was given for 30 minutes. It the duration was changed, the different results might be obtained.

3. Optimal durations of irradiations in utilizing ultrasonic waves

As shown in report 1, previously reported, 120 minute irradiation was apparently too long. So, irradiation was conducted for 5, 15, 30, 60 and 90 minutes. The cycle was limited to 200 and 600 Kc and 1.5 Kc, based on the experimental results which high cycles were more suitable for the good results than low cycles. Hairs were treated by the swollen method and the crushed method in order to study comparatively the optimal duration of irradiation and the relationship with the cycle.

In the swollen method 5 minute irradiation was insufficient at each cycle. Fifteen minutes was adequate at each cycle of 200 and 600 Kc but insufficient at 1.5 Mc, 30 minutes appeared to be adequate at each cycle, 60 minutes appeared to

be little too long at 200 Kc but adequate at 600 Kc and 1.5 Mc. Ninty minutes was apparently too long at both 200 and 600 Kc but sufficient at 1.5 Mc as same as 30 minutes. In the crushed method 5 minute irradiation was insufficient, 15 minutes was adequate at each cycle between 200 and 600 Kc but slightly insufficient at 1.5 Mc, 30 minutes was a little too long at 200 and 600 Kc and adequate at 1.5 Mc, and 60 or 90 minutes at 600 Kc and 1.5 Mc was the same as 30 minutes. Ninty minutes was apparently too long at 200 Kc.

The fact that a long term irradiation at a low cycle gives rise to a type non-specific reaction might be explained by adsorbing even non-corresponing anti-serum through sustained intense ultrasonic action even after the completion of reaction within a short time. The ultrasonic caviation and vibration are seen to cause these phenomena, as previously reported. The ultrasonic irradiations make the hairs, destroyed to some degree by preliminary treatments, more destructive and make the surface of the hairs rough extremely. At the same time it seems that the irradiations give rise to the adhesive phenomenon of excessive blood cells which do not play a part in the antigen antibody reaction. Based on these observations, the short time irradiation with relative high cycles appears to be adequate for the mixed agglutination test and 15 minutes appears to be the adequate duration of irradiations.

4. Relationship between preliminary treatments and ultrasonic wave irradiations having influence on the absorption of antibodies by hairs

The optimal cycles and duratins of irradiations utilizing ultrasonic waves were studied. It seemed that 15 minute irradiation at each cycle between 200 and 600 Kc was adequate for the mixed agglutination test. In order to utilize the most intense cavitation, 200 Kc was chosen.

Hairs subjected to swelling, crushing and decolorizing were irradiated with 200 Kc ultrasonic waves for 15 minutes along with the untreated controls. The agglinations in the swollen method were more intense than that of the untreated controls and the method did not give rise to the type non-specific reaction. The type determination was most definite. On the other hand, the agglutinations in the crushed method were more intense than that of the untreated controls but this method gave rise to the type non-specific reaction and the type determination was apt to be indefinite. The decolorized method gave rise to the type non-specific reaction and the type determination was impossible. Since the method of testing was different, the method of preliminary treatment of hairs should also be studied. Since the technic of blood type determination is different between the elution test and the mixed agglutination test despite similar principles, the preliminary treatment must be carefully examined. In the previous part I, the various methods of preliminary treatment were compared. Even after the introduction of ultrasonic wave irradiation, the specific nature of each method of preliminary treatment was maintained and the irradiation worked effectively. In the mixed agglutination

test like the elution test the differences in intensity were noted between reactions of anti-A and anti-B serum. These are probably due to a common characteristic in anti-A serum,[15] as was pointed out in the previous report I. As described above, in the mixed agglutination test, the best result was obtained in the hair treated by the swollen method. It appeared possible to shorten the time of absorption from 3 hours to 15 minutes by utilizing ultrasonic wave irradiations during the antibody absorption period, compared with the conventional non-irradiated test. The blood type determination was more definite. This method, described above, appears to be sufficiently useful for the blood type determination of hairs in the practical field of legal medicine.

CONCLUSION

The mixed agglutination test by the use of ultrasonic wave irradiations appears to be more useful for the blood type determination of hairs than the conventional non-irradiated test. The advantages were shortening of the absorption time and clarification of blood types. The best result was obtained in the swollen method as the preliminary treatment of hairs, 200 Kc as the optimal cycle and 15 minutes as the optimal duration during the antibody absorption period.

REFERENCES

1) Komura, S. et al.: *Bull. Yamaguchi Med. School*, **16**: 91, 1969.
2) Coombs, R. R. A. et al.: *Vox Sanguinis*, **5**: 111, 1955.
3) Coombs, R. R. A. et al.: *Med. Sci. Law*, **1**: 359, 1961.
4) Oyama, T.: *Nagasaki Igk.*, **2**: 40, 568, 1965.
5) Kuwahara, H.: *Nagasaki Igk.*, **2**: 42, 1046, 1967.
6) Yada, S.: *Jap. J. Legal Med.*, **15**: 98, 1961.
7) Coombs, R. R. A. et al.: *Lancet*, **270**: 461, 1956.
8) Nakata, K.: *Acta Crim. Jap.*, **25**: 1959.
9) Yada, S. et al.: *Jap. J. Legal Med.*, **16**: 233, 1962.
10) Murakami, S. et al.: *Jap. J. Legal Med.*, **18**: 206, 1964.
11) Akaishi, S. et al.: *Jap. J. Legal Med.*, **19**: 181, 1965.
12) Oyama, T.: *Nagasaki Igk.*, **2**: 42, 888, 1967.
13) Yasuda, N. et al.: *Hoi Kansiki Syakai Igaku Zassi*, **3**, **4**: 31, 1957/1958.
14) Zitsuyoshi, Z. et al.: *Choonpa Gijutsu Binran*, Nitsukan Kogyo Shinbunsha, Tokyo, 1966.
15) Kitahama, M. et al.: *Reports of the National Research Institute of Police Science*, **21**: 106, 1968.