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Antigenic Determinants of the W28 Molecule Different from but Spatially Close to W28 Determinant

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The existence of two allelic determinants, subtypic to W28 (named TO 54 and TO 55), and of an antibody cross-reacting W28 × HL–A10, is demonstrated. These new determinants have been studied by means of population and family studies and with the following serological techniques: absorption, elution, lysostrip and blocking by $F(ab')_2$ fragment. The results suggest that determinants TO 54 and TO 55 are very similar each other and are spatially close to the supertypic factor.

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It has been shown that the product of a single HL–A allele possesses more than one antigenic determinant (Bernoco et al. 1973, Legrand & Dausset 1973). Some of these determinants are "public" or "supertypic" (Mi & Morton 1966), i.e. common to several different allelic products. For example, antigen 8a (van Rood et al. 1965) is present on two HL–A factors, HL–A2 and W28, which in turn are "subtypic" to 8a (van Rood et al. 1970). Recent evidence demonstrates that some subtypic specificities are supertypic to others, so that the distribution pattern of some groups of antigens in the population is similar to a "chinese box" (Ceppellini et al. 1967). As studies on HL–A antigens increase, more and more specificities are identified on each HL–A molecule. Preliminary studies on the spatial arrangement of different determinants on the same molecule, performed by means of either cytotoxicity (Richiardi et al. 1973) or of adsorption inhibition (Legrand & Dausset 1973), lead us to believe that they are located in different areas of the molecule, so that the respective specific antibodies are sufficiently separated not to reciprocally hinder one another. Studies on H2 antigens (Cresswell & Sanderson 1968) on the other hand, give evidence that some determinants are so close together that the attachment of an antibody to a determinant sterically inhibits adsorption of a second antibody specific for a different, closely located determinant.

Results presented here concern the identification of two allelic factors subtypic to

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W28, previously thought to be homogenous in the population. Evidence is presented that these subtypic determinants are located in close proximity to a supertypic determinant. Furthermore, results show incomplete blocking of HL-A determinants by cross-reacting antibodies.

Materials and Methods

Sera Studied

Serum RM4 was obtained from an individual immunized by a planned series of 10 intradermal leukocyte and platelet suspension injections. HL-A typing of donor and recipient are shown in Table 1.

Sera 3.23 and 8.20 were obtained by planned whole blood transfusions (Curtoni et al. 1972) (Table 1).

Serological Techniques

a) *Typing* was done by the standard complement-dependent lymphocytotoxic (CdL) test using the NIH micromethod (Brand et al. 1970).

b) *Adsorption.* Serum was incubated with platelet pellets (1 h at +37° C and then overnight at +4° C). One ml of serum adsorbed different platelet concentrations ranging from 0.5×10^9 to 16×10^9.

c) *Elution.* Antibodies adsorbed on platelets were eluted with a modification of the Luzzati et al. (1971) method. Platelets were washed four times with cold phosphate buffered saline (PBS) at pH 8.0, and then resuspended in glycine-HCl buffer 0.5 M pH 3.0 + 1 % bovine serum albumin (BSA). A volume of 0.2 ml acid buffer per ml of adsorbed antiserum was used. After 10 min at room temperature the suspension was centrifuged at 10,000 *g* for 10 min at +4° C. The supernatant was separated and dialyzed against PBS.

Adsorptions and eluates were stored at — 35° C.

d) *Specific resistance to lysis* (lysostrip) (Bernoco et al. 1973): lymphocytes were first sensitized (1×10^6/ml antiserum) for 2 h at room temperature by the anti-HL-A sera under study (normal human AB serum was used as control). The coated cells were then washed three times in Hanks' solution and incubated with an anti-human-immunoglobulin serum for 1 h at +37° C. The cells were then washed, suspended in McCoy's medium and incubated for another 20 min at +37° C, re-washed and finally suspended in a 3000/microliter concentration of McCoy's. Then lymphocytes were tested in cytotoxicity. All steps, except for lymphocytotoxicity testing, were carried out in the presence of cycloheximide (50 μg/ml) in order to prevent protein synthesis.

Table 1

HL-A typing of immunized volunteers and their blood donors

	LA	FOUR	AJ	4a, 4b	Serum studied
Donor (RS)	HL-A2, W28	HL-A5, W5	T4	4a 4b	RM4
Recipient (LG)	HL-A2, HL-A3	W18, W5	ND	4b	
Donor (CR)	HL-A9, W26	W18, W21	T6	4a 4b	3.23
Recipient (BL)	HL-A2, HL-A3	W10	ND	4b	
Donor (MM)	HL-A1, HL-A2	HL-A5, W18	ND	4a 4b	8.20
Recipient (GP)	HL-A3, W32	HL-A7, W18	ND	4b	

ND: not determined.

e) *Inhibition test with $F(ab')_2$ fragment:* $F(ab')_2$ fragment was prepared by treating the serum-isolated Ig fraction with pepsin. This technique has already been described in detail elsewhere (Richiardi et al. 1973). The test was performed by incubating lymphocytes with the $F(ab')_2$ fragment for 2 h at +4° C (2000 cells/microliter). The suspension was then centrifuged and the cells suspended in McCoy's medium and tested against different cytotoxic sera.

f) *Panel of test lymphocytes.* Sera were tested with a panel of lymphocytes which had been typed for the following HL-A specificities:

a) LA series: HL-A1, 2, 3, 9, 10, 11, W23, W24, W25, W26, W28, W29, W30, W31, W32.
b) FOUR series: HL-A5, 7, 8, 12, 13, W5, W10, W14, W15, W16, W17, W18, W21, W22, W27.
c) AJ series: T1, T2, T3, T4, T5, T6 (for nomenclature, see Mayr et al. 1973). Not all panel cells had been typed for these antigens.
d) 4a and 4b.

Antigen W28 was defined with the following sera: Boccard 555 (Dr. Jeannet), Harris 2.50.603.3001 and Harris 2.50.6.04.2201 (from NIH), CLB 29 (Dr. Engelfriet), 34.24 (Turin). All these sera were tested against a panel of 400 individuals of the normal Italian population and they all reacted strongly against W(28+) cells; both Harris samples and CBL 29 generally do not react with HL-A(2+) cells: the few exceptions encountered were mainly HL-A2-homozygous. Due to these typing difficulties, only HL-A(2+),W(28+) cells ascertained through family studies have been considered (Table 5). This limitation does not permit frequency calculations.

Results

Reaction pattern of serum RM4 in the immunizer's family. The HL-A typing of the immunizer's (RS) family is shown in Table 2. All family members have been tested with serum RM4: both father and mother react positively with the serum, though at a different titer (father 1:2, mother 1:8). The serum titer against RS lymphocytes is 1:16. The family pattern shows that the serum react against the paternal haplotype "b" (HL-A2,5,4a) and the maternal haplotype "d" (W28,W5,4b,T4). Checkerboard adsorptions between family members (Table 3) show that antibody activity against the two immunizer haplotypes can

Table 2

Serum RM4: HL-A typing of the immunizer's (RS) family

	LA	FOUR	AJ	4a, 4b	Serum RM4 titer	Haplotypes
Father	HL-A2	HL-A5	T6	4a 4b	1 : 2	ab
Mother	W28, W30	HL-A8, W5	T4, T6	4b	1 : 8	cd
Sibling 1	HL-A2 W30	HL-A8	T6	4b	–	ac
Sibling 2 (RS)	HL-A2, W28	HL-A5 W5	T4	4a 4b	1 : 16	bd
Sibling 3	HL-A2, W28	HL-A5 W5	T4	4a 4b	1 : 16	bd
Sibling 4	HL-A2 W30	HL-A8	T6	4b	–	ac

Haplotypes: a: HL-A2 T6 4b; b: HL-A2 HL-A5 4a; c: W30 HL-A8 T6 4b; d: W28 W5 T4 4b

Table 3

Serum RM4 absorption studies with the immunizer's family

		Lymphocytotoxicity test with cells:			
		Father	Mother	Child 2 (immunizer)	Child 1
	Haplotypes	ab	cd	bd	ac
Serum RM4 unadsorbed		2*	8	16	–
Serum RM4 adsorbed with platelets from:					
Father	ab	–	8	8	–
Mother	cd	2	–	2	–
Sibling 2 (immunizer)	bd	–	–	–	–
Sibling 1	ac	2	8	16	–

* Numbers indicate reciprocal of serum titer.

Table 4

Resistance to lysis (lysostrip) induced by serum RM4 on immunizer's cells

	Typing after lysostrip				
	HL–A2	W28	HL–A5	W5	T4
Cells pre-treated with:					
Normal AB serum	+	+	+	+	+
Serum RM4	+	–	–	+	+
Serum RM4 adsorbed with:					
paternal platelets	+	–	+	+	+
maternal platelets	+	+	–	+	+

easily be separated. This result is confirmed by lysostrip experiments performed on immunizer cells (Table 4). Serum RM4 can aggregate and remove antigens W28 and HL–A5; when adsorbed with RS paternal platelets (haplotype "b") it removes W28; when adsorbed with maternal platelets (haplotype "d") it removes HL–A5. Antigen T4 is not affected by serum RM4.

Only antibodies against maternal haplotype "d" will be considered here. All experiments presented (Tables 5 to 11) have been performed using antiserum previously adsorbed with paternal platelets.

Population studies. Serum RM4, tested against a panel of 400 donors, reacted positively with all HL–A(10+) and W(28+) lymphocytes. All HL–A(10+) cells equally reacted with this serum, while two different reaction patterns could be distinguished in W(28+) cells (Table 5). The W(28+) cells reacting strongly (1:8) with serum RM4 are believed to possess a new specificity called "TO 54". The W(28+) individuals yielding weak, non-reproducible reactions with this serum have been named "TO 55".

Family studies show that the strength of the reactivity of serum RM4 is genetically determined and segregates with the W28 carrying haplotype both when it is strong

Table 5

HL-A phenotypes of a panel of W(28+) cells, and serum RM4 titer

Cell No.	LA		FOUR		AJ		4a	4b	Serum RM4 titer
14073	W30	W28	HL-A8	W5	T4	T6	4a	4b	1 : 8
13459	HL-A9	W28	W5		ND		4a	4b	1 : 8
77	HL-A3	W28	W10		T6			4b	1 : 8
14535	HL-A2	W28	HL-A12	W5	ND		4a	4b	1 : 8
1030	HL-A1	W28	HL-A5	W17	-		4a		1 : 1
3026	HL-A1	W28	HL-A8	W15	ND			4b	1 : 1
10365	HL-A1	W28	HL-A8	W5	T4			4b	1 : 1
13294	W32	W28	HL-A12	W15	T5		4a	4b	1 : 1
13126	HL-A2	W28	W5		T2	T4	4a	4b	1 : 1
170	W32	W28	HL-A5	W27	T2	T6	4a		1 : 1
14440	HL-A1	W28	HL-A8	W16	T6			4b	1 : 1
14190	HL-A2	W28	HL-A8	W16	T6			4b	1 : 1
14517	HL-A3	W28	HL-A13	W5	ND		4a	4b	1 : 1

ND: not determined.

Table 6

W28 (TO54 and TO55) haplotypes

Family no.	Haplotypes		No. of children	No. of children with W28 haplotype	Titer of RM4 with members W(28+)
	Paternal	Maternal			
381	W28 (TO55), W5/9, 8	W32, W14/2, -	7	3	1/1
453	1, W17/3, W15	W28 (TO55), 5/2, -	3	2	1/1
389	W28 (TO54), -/9, W5	3, W5/W30, -	5	1	1/8
413	2, -/2, 5	W28 (TO54)/W30, 8	4	2	1/8
42	W28 (TO54), W16/1, W5	9, W21/3, W10	6	3	1/8
Total			25	11	

and when it is weak. At the present time, five large families, three of which are TO (54+) and two are TO (55+) have been typed (Table 6).

Adsorption and elution studies of serum RM4 (Tables 7 and 8) were performed to investigate the relationship between anti-TO 54, TO 55, and HL-A10 antibody activity.

Quantitative adsorption studies (Table 7) show that both TO(54+) and TO (55+) platelets can, at varying concentrations, completely adsorb cytotoxicity against all W(28+) cells. On the contrary, HL-A(10+) platelets cannot adsorb anti-TO 54 antibodies even at a concentration of 10×10^9/ml.

When absorbed either with TO(55+) or HL-A(10+) platelets, the serum shows the same reaction pattern against TO (55+) and HL-A(10+) lymphocytes: 0.5×10^9/ml platelets are sufficient to adsorb all cytotoxic activity.

Eluates from either TO(54+), or TO (55+) or HL-A(10+) platelets all react identically against TO 55 and HL-A10 lymphocytes (Table 7). On the contrary, the reactivity against TO(54+) cells is stronger.

Table 7

Quantitative serum RM4 adsorption studies

Test with lymphocytes**	Adsorbing dose (x10⁹/ml)	Adsorbed with platelets			
		W28	+	+	−	−
		TO54	+	−	−	−
		TO55	−	+	−	−
		HL-A10	−	−	+	−
TO(54+)	0.5		1*	4	4	8
	1		1	2	4	8
	2		−	1	2	8
	4		−	1	2	8
	8		−	−	2	8
	16		−	−	2	8
TO(55+)	0.5		−	−	−	1
	1		−	−	−	1
	2		−	−	−	1
	4		−	−	−	1
	8		−	−	−	1
	16		−	−	−	1
HL-A(10+)	0.5		−	−	−	1
	1		−	−	−	1
	2		−	−	−	1
	4		−	−	−	1
	8		−	−	−	1
	16		−	−	−	1

* Numbers indicate the reciprocal of serum titer. Each is the result of three different absorption experiments.

** The test lymphocytes are cells nos. 14073, 77, 1030, 3026, 10365 shown in Table 5, and three HL-A(10+) cells.

Table 8

Elution studies of serum RM4

				Test with lymphocytes		
				TO(54+)	TO(55+)	HL-A(10+)
Unadsorbed serum				8*	1	1
Eluates from 8 × 10⁹ platelets:						
W28	TO54	TO55	HL-A10			
+	+	−	−	8	1	2
+	−	+	−	4	1	2
−	−	−	+	2	1	2
−	−	−	−	−	−	−

* Numbers indicate reciprocal of serum titer.

Note: eluates were made from two different platelet samples for each specificity and tested with a panel of lymphocytes.

These experiments lead to the conclusion that the serum contains: 1) antibodies cross-reacting with TO 54 and TO 55 (TO 55 reactivity is CYNAP: cytotoxicity negative, adsorption positive); 2) antibodies cross-reacting with TO 54,TO 55 and HL-

Table 9

Antibodies cross-reacting with HL-A10 and W28 in serum 3.23: adsorption and elution studies

			Test with lymphocytes	
			W(28+)	HL-A(10+)
Unadsorbed serum			1*	16
Serum adsorbed with platelets:	W28	HL-A10		
	+	−	−	8
	−	+	−	−
	−	−	1	16
Eluate from platelets:	+	−	2	2
	−	+	1	32
	−	−	−	−

* Numbers indicate reciprocal of serum titer.

Table 10

Resistance to lysis after treatment with whole or adsorbed serum RM4

Lymphocytes	HL-A antigens	Pretreatment with serum RM4		
		Unadsorbed	Adsorbed with TO(55+) platelets	Adsorbed with HL-A(10+) platelets
TO(54+)				
	HL-A3	0	0	0
	W28	100	0	100
	W10	0	0	0
TO(55+)				
	HL-A1	0	0	0
	W28	70	0	10
	HL-A8	0	0	0
	W17	0	0	0
HL-A(10+)				
	HL-A2	0	0	0
	HL-A10	100	0	0
	W16	0	0	0

Numbers indicate the resistance to lysis percentage observed when cells are typed for the corresponding antigens.

A10. This demonstrates the presence of a new type of cross-reaction: W28×HL-A10. This result is also confirmed by *experiments performed on serum 3.23,* which reacts with all HL-A(10+) (titer 1:16) and W(28+) (titer 1:1) lymphocytes. Adsorption and elution experiments (Table 9) show that the serum contains two antibody populations: 1) anti-HL-A10 antibodies; and 2) HL-A10 and W28 cross-reacting antibodies.

Lysostrip experiments with serum RM4 were performed on either TO(54+), or TO(55+) or HL-A(10+) lymphocytes (Table 10). Treatment with serum RM4 induces 100 % resistance to lysis by anti-W28 antisera on TO(54+) lymphocytes;

the same same result is obtained on HL–A (10+) lymphocytes. TO(55+) cells results shows them to be only 70 % resistant to lysis.

Serum RM4 adsorbed with 8×10^9 TO (55+) platelets cannot remove TO 54,TO 55 or HL–A10 from the cell surface. When adsorbed with HL–A(10+) platelets it still prevents lysis of TO(54+) lymphocytes. On the other hand, it has almost no effect (10 % resistance to lysis) on TO (55+) cells.

Blocking of W28 with $F(ab')_2$ fragment from serum RM4. Results are presented in Table 11. There is only a partial inhibition against TO(55+) cells, while anti-W28 sera are completely inhibited by $F(ab')_2$ fragment of serum RM4 against TO(54+) lymphocytes.

Serum 8.20 (see Table 1) reacts in CdL against all HL–A(1+), HL–A(2+) and W(24+) lymphocytes. Elution studies demonstrated that it contains 1) antibodies cross-reacting with HL–A1 and W24; and 2) antibodies cross-reacting with HL–A2, W24 and W28. Cytotoxic activity against W28 can be seen only in the eluates. Eluates from W24,HL–A2 and HL–A1 platelets have been tested against both TO (54+) and TO(55+) lymphocytes (see

Table 11

Inhibition (%) by $F(ab')_2$ fragment from serum RM4

	Inhibition percentage of						
	Serum RM4	Sera anti					
		HL–A1	HL–A3	W28	HL–A8	W10	W17
TO(54+) cells:							
uncoated	0		0	0		0	
coated with $F(ab')_2$	100		0	100		0	
TO(55+) cells:							
Uncoated	0	0		0			0
coated with $F(ab')_2$	100	0		46	0		0

Table 12

Elution studies of serum 8.20

				Test lymphocytes			
				HL–A(2+)	W(24+)	HL–A(1+)	W(28+)
Unadsorbed serum				+	+	+	–
Eluates from platelets:							
HL–A2	W24	HL–A1	W28				
+	–	–	–	+	+	–	+
–	+	–	–	+	+	+	+
–	–	+	–	–	+	+	–
–	–	–	+	+	+	–	+

Note: eluates were made from three different platelet samples for each specificity and tested with a panel of lymphocytes, always with the same results.

Table 13

Serum 8.20 elution studies with test lymphocytes TO(54+) and TO(55+)

	Test with lymphoctyes	
	TO(54+)	TO(55+)
Eluates from platelets:		
W(24+)	30*	66
HL-A(2+)	50	72
HL-A(1+)	0	0

* Numbers represent scores of antibody reactions calculated by summing of positive reaction strengths from serial titrations:
80–100% killed cells = score 8
60– 80% killed cells = score 6
40– 60% killed cells = score 4
20– 40% killed cells = score 2
Each score sums the results of three different experiments.

Table 12). Each eluate is more weakly positive against TO(54+) than TO(55+) cells.

Unfortunately, the limited amount of serum available did not enable further studies to be performed.

Discussion

Experiments demonstrate that anti-W28 activity of serum RM4 has a different pattern against all W(28+) samples tested. The reaction is stronger against some W (28+) samples (called TO 54) and weaker against the rest (called TO 55). Adsorption and elution studies show that serum RM4 has two antibody populations, both of which react against TO 54 and TO 55: a population cytotoxic for all cells having one of the three TO 54,TO 55,HL-A10 factors, and a population cytotoxic for TO 54 and CYNAP for TO 55. Both these antibodies have a better reaction with TO 54 than TO 55.

When W(28+) cells are tested with eluates from serum 8.20 (eluates, having a higher antibody concentration, are cytotoxic for W28, while the original serum is not), the results are symmetrical to those obtained using serum RM4. Cytotoxic activity has a higher titer against TO(55+) than TO(54+) cells.

Lysostrip experiments and studies with $F(ab')_2$ fragment of serum RM4 confirm the difference between TO 54 and TO 55 factors.

The difference between TO 54 and TO 55 appears genetically determined: three out of five large families studied show allele TO 54, while the other two transmitted TO 55; thus, partition of W28 into two allelic factors, TO 54 and TO 55, is consistent in families (see Table 6).

Several hypotheses can explain the different reaction pattern shown by serum RM4 against TO 54 and TO 55. A first hypothesis considers TO 54 and TO 55 determinants structurally identical but having different density on the cell surface; lower on TO(55+) than on TO (54+). A lower density could account for the lower antibody titer, demonstrating that the cytotoxic titer of an antibody is influenced by the density of the antigenic determinants on the cell surface (Linscott 1970). The lower the antigen density, the lower the probability that in a given incubation time an antibody population finds all antigenic determinants and binds them; this could explain partial inhibition by the $F(ab')_2$ fragment and incomplete resistance to lysis induced on TO(55+) cells.

On the other hand, data obtained using other antisera are not consistent with this hypothesis. Typing anti-W28 sera react equally with both TO(54+) and TO (55+) cells. This is in favour of an equal distribution of W28 determinants in all W(28+) cells. Since TO 54 and TO 55 are produced by the same LA allele that controls W28 (as shown by lysostrip experiments and by family segregation), the

different density hypothesis implies that TO(55+) cells present two types of W28 factors, one of which carries TO 55 determinants. Another argument against this hypothesis arises from serum 8.20 results. Its antibody activity is stronger against TO 55 than TO 54; this is exactly the opposite to that observed with serum RM4. Serum 8.20 results should demonstrate a lower antigen density of TO(54+) cells than TO (55+), which is obviously contradictory to the hypothesis arising from serum RM4 studies.

For these reasons, it seems unlikely that TO 54 and TO 55 differ in their distribution on the cell surface and not in their structure.

A second hypothesis is that TO 54 and TO 55 are structurally different, though similar, determinants, so that an antibody produced by stimulation with TO(54+) cells can also react against TO 55, but with less avidity. ("Avidity" indicates "an operationally defined estimate of binding strength" (Siskind & Benacerraf 1969)). Many examples are known of an antibody showing higher affinity for the immunizing antigen than for cross-reacting determinants (Pappenheimer et al. 1968, Speyer et al. 1973).

A lower avidity of the anti-TO55 in respect to anti-TO 54 activity in serum RM4 could explain all the results obtained. Antibodies with low avidity towards the TO 55 determinant would not bind very strongly to the antigen and could easily detach during incubation. This could account for the lower cytotoxic titer, the greater amount of TO(55+) (compared with TO (54+)) platelets needed to absorb all antibody activity from serum RM4, and for incomplete resistance to lysis induced on TO(55+) lymphocytes. The latter is not due, in this case, to the fact that TO 55 and W28 determinants belong to different genic products and therefore are on different molecules (Bernoco et al. 1973) (unlikely from the data), but to a low avidity of anti-TO 55.

$F(ab')_2$ results can also be explained this way. Another phenomenon may play a role here: low avidity antibody fragments (anti-TO 55) can be detached by higher-avidity anti-W28 antibodies used in the test.

As mentioned above, serum RM4 contains two different types of antibodies: 1) a population cytotoxic for TO 54, TO 55 and HL–A10, that can be purified by elution from HL–A(10+) platelets; and 2) a population cytotoxic against TO 54 and CYNAP for TO 55, that can be obtained by adsorption with HL–A(10+) platelets. These two antibody classes can be separately studied. Cytotoxic activity of serum is due only to type 1) antibodies, and not to a synergistic effect of two antibody populations (Ahrons & Thorsby 1970); the CYNAP phenomenon is shown only by type 2) antibodies. Table 9 shows lysostrip experiments with whole serum RM4 and serum RM4 previously adsorbed with HL–A(10+) platelets. The ability to induce resistance to lysis varies considerably in the two experiments: the activity of anti-TO 55 CYNAP antibody is much lower than that of the cytotoxic anti-TO 55. This difference concerns only determinant TO 55: in fact, both anti-TO 54 antibody populations show the same resistance-inducing ability. Thus, the difference in this serum between CYNAP and cytotoxic behaviour appears due to a difference in avidity.

In conclusion, TO 54 and TO 55 regions are probably two different allelic products, subtypic to W28, which can be distinguished by different reaction patterns with the same antibody. Even though different, they are strongly analogous: in fact antibodies specific for either TO 54 or TO 55 alone have not as yet been found. These two subtypic factors thus appear different from

others already described, such as W25 and W26 (Richiardi et al. 1971), TO 52 and TO 53 (Richiardi et al., in press). In these cases, activity against two subtypic factors can be split into two different antibody populations; on the contrary, activity against TO 54 and TO 55 is carried by the same antibody molecule. Another difference between subtypic specificities TO 54, TO 55 and subtypic W25,W26 arises from $F(ab')_2$ fragment experiments (Richiardi et al. 1973). If HL–A(10+) cells are coated by a $F(ab')_2$ from an anti-W25 serum, they are still susceptible to lysis by anti-HL–A10 sera, and vice versa. The same has been proven in experiments with different supertypic antibodies and the corresponding subtypic determinants. This would mean that supertypic and subtypic determinants of the same HL–A molecule are located at a sufficient distance to prevent "steric hindrance". This is not so for TO 54,TO 55 and W28. $F(ab')_2$ fragments from serum RM4 can inhibit the cytotoxic action of anti-W28 sera, completely on TO(54+) and partially on TO (55+) cells. Such inhibition means that antibodies of serum RM4 react either 1) exactly against the same determinant which binds the anti-W28 antibodies; or 2) against a region partially common to that of the W28 determinants; or 3) with a determinant distinct from but close enough to W28, so that respective antibodies can reciprocally hinder each other. It does not seem probable that serum RM4 reacts with the same exact region against which other anti-W28 sera are directed: in fact, in this case it would probably react the same way with all W(28+) cells. Thus, the case described here probably concerns two subtypic specificities which, contrary to those hitherto studied, are very closely located near a supertypic determinant.

Another uncommon aspect of serum RM4 is the presence of a cross-reaction between W28 and HL–A10. This cross-reaction is also confirmed by serum 3.23 (Table 8) and has not as yet been described elsewhere. Generally, W28 is known to cross-react with HL–A2 and 9, while HL–A10 cross-reacts with 1, 11 and W19. The most immunogenic part of the W28 molecule appears similar to HL–A2. In fact, many antibodies are known to react with all HL–A(2+) and all W(28+) cells, and many antibodies specific for W28 are also cytotoxic for some HL–A(2+) cells, particularly those belonging to individuals homozygous for HL–A2. The producer of serum RM4 could not produce antibodies against the part of W28 region cross-reacting with HL–A2, being HL–A(2+) himself. He became sensitized against the other part of the W28 molecule only after 10 intradermal injections of buffy coat suspensions, a number higher than that usually necessary to induce immunization (Curtoni et al. 1972). This shows that the TO 54 determinant is relatively poorly immunogenic.

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