

# ANTIBODY RESPONSES TO ANTIGENIC DETERMINANTS OF INFLUENZA VIRUS HEMAGGLUTININ

## II. Original Antigenic Sin: A Bone Marrow-Derived Lymphocyte Memory Phenomenon Modulated by Thymus-Derived Lymphocytes

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The "original antigenic sin" phenomenon (OAS)<sup>1</sup> challenges the dogma of the specificity of the immunological memory: when there is sequential infection with two different but antigenically related strains of influenza A virus, the antibody stimulated by the second infection reacts more strongly with the primary virus than with the one actually eliciting the response (1-3). This phenomenon is now well documented with many viral and nonviral cross-reacting antigens (4-8). However, its study with influenza antigens is of special interest because of its wide implications in the sero-epidemiology of influenza and the response to vaccination. In the accompanying paper (9) we have shown that purified hemagglutinin (HA) extracted from related influenza viruses share cross-reacting antigenic determinants, but differ in strain-specific determinants. We have now analyzed the antibody response to each of these groups of determinants after sequential exposure of mice to two related HA's, and have carried out cell-transfer experiments in an attempt to elucidate the cellular mechanisms responsible for the aberrant immunological recall of the OAS phenomenon.

### Materials and Methods

*Animals, Immunization Schedules, Preparation of Purified-Concentrated Viruses, Bromelain-Extraction of HA.* The animals, immunizations, etc., and the independent measurement of

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<sup>1</sup> *Abbreviations used in this paper:* CR, cross-reacting population of antibody; H<sub>1</sub>, cross-reacting; H<sub>3</sub>, unrelated hemagglutinin; HA, purified hemagglutinin; Ho, homologous; OAS, original antigenic sin; S<sub>1</sub>, strain-specific antibodies to H<sub>1</sub> hemagglutinin; So, strain-specific antibodies to Ho hemagglutinin; SRDT, single radial diffusion tests; T<sup>+</sup> mice, immunologically intact mice; TXBM mice, thymectomized, irradiated, and bone marrow reconstituted mice.

antibody to strain-specific and cross-reacting determinants of the HA molecule by single-radial immunodiffusion have been described in the accompanying paper (9).

*Preparation of Spleen Cell Suspensions.* Single-cell suspensions were obtained by gentle forcing through a stainless steel sieve; they were then washed, and resuspended in L 15 medium.

*Anti- $\theta$  Serum.* AKR anti- $\theta$  C3H serum and fresh rabbit serum were separately absorbed twice with an equal volume of a fresh liver suspension collected from normal CBA mice, for 1 h at room temperature. A mixture of 1 vol of packed spleen cells, 1 vol of undiluted fresh rabbit serum and 2 vol of anti- $\theta$  serum diluted 1:10 (final dilution 1:20) was incubated at 37°C for 1 h with intermittent gentle mixing, washed twice, and resuspended in L 15 medium. The viability of these treated spleen cell suspensions was about 60%–65% before transfer.

## Results

### *Responses of Ho-Primed Mice to Homologous or Heterologous Boosting.*

Groups of mice were primed with three injections of 10  $\mu$ g Ho hemagglutinin (bromelain-extracted from influenza A/PR8 virus) and left for 2 mo without further immunization. The animals were then boosted with 10  $\mu$ g of either homologous (Ho), cross-reacting (H<sub>1</sub>), or unrelated (H<sub>3</sub>) hemagglutinin. The arithmetic mean of antibody titers for each group was calculated in the sera collected 7, 14, and 21 days after the boost and tested for potency and specificity in single-radial-diffusion immunoplates (SRDT). Unprimed animals did not give any detectable response, 10  $\mu$ g of either Ho or H<sub>1</sub> being below the threshold immunogenic dose of these antigens. Fig. 1 shows that the H<sub>3</sub> boost did not modify the progressive antibody decrease observed 2 mo after priming. The Ho boost recalled the two antibody populations corresponding to the two groups of

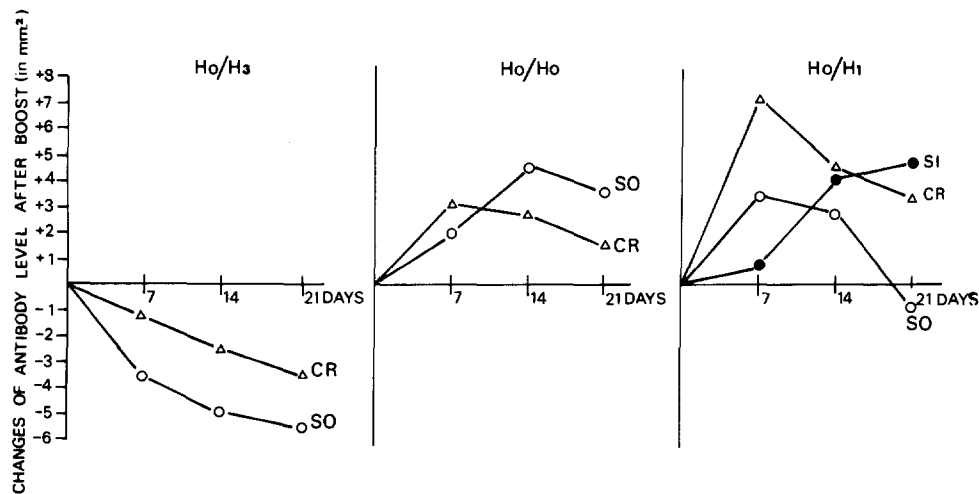


FIG. 1. Secondary response curves of antibodies to different antigenic determinants of bromelain-extracted HA molecule in Ho-primed mice boosted with 10  $\mu$ g of either H<sub>3</sub> (Ho/H<sub>3</sub> sequence), Ho (Ho/Ho sequence), or H<sub>1</sub> (Ho/H<sub>1</sub> sequence). Changes in antibody titers are expressed as differences (in mm<sup>2</sup> of zone areas) between arithmetic mean of titers for groups of 10 mice before the boost and 7, 14, and 21 days after the boost. The specificity and potency of each antibody population were determined by cross-absorption in single-radial diffusion immunoplates, as described in the accompanying paper.

antigenic determinants present in the Ho molecule (So, strain-specific and CR, cross-reacting) as expected. Paradoxically, the H<sub>1</sub> boost was also followed by a secondary-type antibody response of the So specificity, and thus specific for antigenic determinants which are not present in the boosting antigen. A third antibody population was found in the latter group (Ho/H<sub>1</sub> sequence), which was not absorbable by A/PR8 virus in SRDT containing A/FM1 virus (S<sub>1</sub> population, strain-specific for H<sub>1</sub>). The kinetics of the response of the S<sub>1</sub> population appeared to be intermediate between these typical of a primary and a secondary humoral response, since 10 μg of H<sub>1</sub> is not an immunogenic dose in virgin animals.

*Transfer of Ho-Primed Memory Cells.* Spleen cells from Ho-primed mice were transferred into irradiated recipients either as a whole population (after *in vitro* treatment with a non-T-cell cytotoxic normal mouse serum and complement), or as a T-cell-depleted population (after treatment with anti-θ serum and complement). Table I shows that the recipient animals injected with either Ho or H<sub>1</sub> had a secondary response of both the So and the CR specificity, even when the spleen cells were transferred after anti-θ serum treatment. The amount of antibody secreted was the same whether or not primed T cells were transferred together with the primed B cells, except in the case of the recipients boosted with Ho which produced almost three times more So antibodies after transfer of untreated spleen cells.

Table II shows the results of an experiment involving transfer of anti-θ treated Ho-primed spleen cells into either normal (T<sup>+</sup>) or thymus-deprived (TXBM) recipient mice. Again, a successful adoptive transfer of So-specific and CR-specific memory cells was found, even following transfer of spleen cells collected 6

TABLE I  
*Antibody Responses of Irradiated Mice After Transfer of HO-Primed Spleen Cells Either Treated or Not with Anti-θ Serum and C*

Irradiation of recipient mice*	Spleen cells transferred‡	Treatment of cells in vitro§	Boosting antigen	Anti-SO response¶	Comparison between groups	Anti-CR response¶	Comparison between groups
×600 R	Virgin	0	HO	0	—	0	—
×600 R	0	0	HO	0	—	0	—
×600 R	HO-primed	Anti-θ serum + C	HO	9.2	×2.9	2.5	≈1
×600 R	HO-primed	NMS + C	HO	25.1 (7.0-12.0) (15.0-42.0)		2.0 (0.6-4.4) (2.0-2.0)	
×600 R	HO-primed	Anti-θ serum + C	H <sub>1</sub>	7.6 (7.0-8.8)		4.0 (4.0-4.0)	
×600 R	HO-primed	NMS + C	H <sub>1</sub>	7.6 (5.4-10.7)	≈1	3.9 (2.0-5.4)	≈1

\* Mice were submitted to a 600 rads total body irradiation (×600 R) 4 h before cell transfer.

‡ 1.2 × 10<sup>6</sup> syngeneic spleen cells from a pool of unprimed animals or a pool of animals primed by three injections of 10 μg 2 mo previously were injected i.p. per recipient mouse.

§ Cells were treated by either AKR anti-θ C3H serum (final dilution 1:20) and C or normal CBA mice serum (NMS) and C.

|| Recipients were injected i.p. with 10 μg of either HO or H<sub>1</sub> 24 h after cell transfer.

¶ Sera collected individually 14 days after boosting injections were tested and analyzed for specificity in SRDT plates as described in Methods. Range is indicated between parentheses.

TABLE II  
*Antibody Responses of T<sup>+</sup> or TXBM Mice After Transfer of HO-Primed Spleen Cells Treated In Vitro with Anti- $\theta$  Serum and Complement*

Recipient mice	Spleen cells transferred*	Anti- $\theta$ serum in vitro treatment‡	Boosting antigen§	Determinants specific antibody levels in SRDT (in mm <sup>2</sup> )	
				SO population	CR population
T <sup>+</sup>	Virgin	0	HO	0	0
T <sup>+</sup>	HO-primed	+	0	0	0
T <sup>+</sup>	HO-primed	+	SRBC	0	0
T <sup>+</sup>	HO-primed	+	H <sub>3</sub>	0	0
T <sup>+</sup>	HO-primed 6 mo previously	+	HO	2.9 (2.8-3.5)¶	0
T <sup>+</sup>	HO-primed	+	HO	6.8 (5.4-7.6)	2.5 (1.8-3.0)
TXBM	HO-primed	+	HO	8.0 (5.4-10.0)	2.7 (2.7-2.8)
T <sup>+</sup>	HO-primed	+	H <sub>1</sub>	4.0 (3.9-4.4)	2.0 (1.1-2.6)
TXBM	HO-primed	+	H <sub>1</sub>	6.0 (4.4-7.0)	3.2 (2.6-3.9)

\*  $1.0 \times 10^6$  spleen cells from a pool of animals primed by three injections of 10  $\mu$ g HO were injected per recipient mouse. Cells were collected 2 mo after priming, unless stated otherwise.

‡ Groups marked + received cells previously treated in vitro with AKR anti- $\theta$  C3H serum (final dilution 1:20) and complement.

§ 24 h after cell transfer mice were injected i.p. with either 10  $\mu$ g of H<sub>3</sub>, HO, or H<sub>1</sub>, or  $10 \times 10^6$  SRBC.

|| Sera collected individually 7 days after boosting injection were tested and analyzed for specificity in SRDT plates, as described in Methods.

¶ Range of responses is indicated between parentheses.

mo after priming. Both Ho and H<sub>1</sub>, but not H<sub>3</sub> were able to recall this immunological memory. Paradoxically, the So antibody response was found in repeated experiments to be slightly higher in TXBM than in T<sup>+</sup> recipients.

## Discussion

The experiments reported in this paper show that the original antigenic sin phenomenon is demonstrable at the level of the purified influenza virus hemagglutinin antigens, since Ho-primed mice boosted with the cross-reacting H<sub>1</sub> showed a secondary anti-Ho response. Our results underline the necessity of antigenic relationship between the priming and the boosting antigen in the phenomenon, since the unrelated H<sub>3</sub> was not able to recall this immunological memory. It is hazardous to compare our results obtained in mice using purified viral antigens to the human epidemiological findings. However, data very similar to ours have been obtained in persons previously immune to Ho-bearing viruses (e.g. A/PR8/34) or H<sub>1</sub>-bearing viruses (e.g. A/FM1/47), who did not show any anti-A/PR8 or anti-A/FM1 antibody recall when they were infected with A/Hong-Kong/1/68 virus bearing H<sub>3</sub> hemagglutinin (10, 11).

H<sub>1</sub> hemagglutinin, when boosting Ho-primed animals, elicits a secondary antibody response in both CR and So populations. Thus, antibodies reacting with

the strain-specific determinant of a first hemagglutinin ( $H_0$ ) are produced in response to a second hemagglutinin ( $H_1$ ) which does not contain the same strain-specific determinant. This observation conflicts with the report of Fazekas de Saint-Groth and Webster (2, 3) that every antibody produced after a sequential immunization with cross-reacting strains of influenza A virus was cross-reactive and capable of being absorbed by both strains of virus. However, results very similar to ours have been found with different cross-reactive antigens, such as serum albumins (4), HL-A antigens (5), cross-reacting haptens (6, 7), and streptococcal antigens (8). This "aberrant" antibody recall directed against the specific determinants of the first of two related sequential antigens appears to be a characteristic of the OAS phenomenon.

At the level of the memory cells, such a paradoxical phenomenon has two possible explanations. Either T-memory lymphocytes, with a broader specificity than the B lymphocytes, are triggered by the cross-reacting antigen and are then able to help B-memory lymphocytes to secrete antibody specific for the first antigen, as suggested in other systems (12-14), or B-memory cells with specificity for the first antigen are directly triggered by the cross-reacting antigen. Our results favor the latter hypothesis, since  $H_0$ -primed spleen cells transferred after treatment with anti- $\theta$  serum and complement were able to secrete So antibody in either irradiated or thymus-deprived recipients after a boost with  $H_1$ .

It is conceivable that a small number of residual T cells might have escaped the effect of anti- $\theta$  serum and might have exerted a helper effect in the second host. However, in the case of the  $H_0$ - $H_1$  sequence, no significant difference in antibody titers was observed in irradiated recipients, whether the transferred primed cells had been treated with anti- $\theta$  serum or not (see Table I). This result cannot be attributed to an incomplete activity of antiserum, since the same *in vitro* treatment greatly reduced the secondary antibody response of the same cell preparation in the case of an  $H_0$ / $H_0$  sequence. Thus, B-memory lymphocytes appear to be directly responsible for the OAS phenomenon.

Our finding that  $H_1$  can trigger So memory B lymphocytes is puzzling, since it implies some "error" in antigen recognition by B cells. Receptors for antigen on the surfaces of B lymphocytes are generally believed to be antibody molecules (15). However, findings of Pernis (16) indicate that membrane-fluorescing antibody in some B cells is not of the same class as cytoplasmic fluorescing antibody, suggesting a possible distinction between receptor antibody and secreted antibody. The two might not have precisely the same specificity. Our results lead to the conclusion that receptors for antigen on some B lymphocytes may have broader specificity than the antibody molecules finally secreted by the cells. This interpretation is in agreement with the work by Klinman et al. (17) showing that primed B cells are less fastidious in their affinity requirements for stimulation than precursor B cells, and are not rigorously determinant specific. In our system, it is possible that repeated priming with the strain-specific determinant of  $H_0$  leads to the development of subpopulations of B cells with receptors of broader specificity able to recognize a different but probably structurally similar determinant within the  $H_1$  molecule.

Cooperation between T- and B-memory cells has been shown to occur during the secondary humoral response of mice to foreign erythrocytes (18) as well as in the hapten-carrier system (19). We present evidence that such co-operation occurs during the anti-HA response:  $H_0$ -primed B cells boosted with  $H_0$  in a secondary

host secreted three times more anti-So antibody when transferred together with Ho-primed T cells than when transferred after anti- $\theta$  treatment. However, CR antibody titers were not influenced by the presence of Ho-primed T cells, indicating that T-memory cells had specificity for the strain-specific determinants of the priming hemagglutinin and had provided their helper effect only to So-secreting B-memory cells. Similarly, we have shown in the accompanying paper that help by virgin T cells is required for the So response but not for the CR response. This correlation suggests a quantitative change between virgin and memory T cells, the quality of the co-operation provided to B cells remaining the same. Moreover, animals primed with Ho produced an unusually high titer of primary S<sub>1</sub> antibody when boosted with H<sub>1</sub> antigen. Thus, Ho-primed lymphocytes appear to help virgin B cells to produce antibody against the new antigenic determinant eliciting the primary S<sub>1</sub> response. This is likely to be due to T-memory cells, since anti-Ho B-memory cells should be committed to secrete only anti-So and anti-Cr antibodies. The same interpretation has been drawn by Cunningham and Sercarz (20) for similar results obtained with cross-reacting erythrocytes.

Evidence is given in the present study that T cells can cooperate with B-memory cells in either a positive or a negative way, depending on experimental conditions. While T cells were shown in the accompanying paper to provide a positive helper effect during the primary anti-Ho antibody response, the data now reported show that T cells present in normal recipients were not able to enhance, and indeed seemed to suppress partially, the secondary response of transferred B-memory cells. Preliminary results in our laboratory indicate that IgG antibodies were secreted in the former case while IgM antibodies were secreted in the latter (Virelizier and Allison, manuscript in preparation). This suggests that different kinds of B cells were reacting in these two different situations, and that only IgM-producing cells can secrete antibody independently of T cells. This is discussed further in relation to the nature of the selection involved in the kinetics of the immune responses to influenza HA and the OAS phenomenon.

### Summary

Mice immunized sequentially with two related influenza virus hemagglutinins (HA) produced a secondary antibody response with two different specificities. Some antibodies were specific for determinants common to both HA's. Paradoxically, some antibodies were directed to determinants existing only in the HA first encountered. Primed spleen cells treated with anti- $\theta$  serum and complement were transferred from animals immunized with the first HA to either normal, irradiated, or thymus-deprived recipients. These memory cells were boosted in the recipients with either the homologous or the heterologous cross-reacting HA. B-memory lymphocytes were shown to be directly triggered by both HA's and to be able to secrete, independently of T lymphocytes, antibodies to both kinds of determinants. However, T cells were shown to modulate this secondary response by either enhancing or suppressing antibody secretion by B-memory cells, depending on experimental conditions. These results are discussed in terms of

antigen recognition by B cells and of kinetics of development of immunological memory.

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