ANTIBODY RESPONSES TO ANTIGENIC DETERMINANTS OF INFLUENZA VIRUS HEMAGGLUTININ

I. Thymus Dependence of Antibody Formation and Thymus Independence of Immunological Memory

By JEAN-LOUIS VIRELIZIER,* ROY POSTLETHWAITE,‡ GEOFFREY C. SCHILD, and ANTHONY C. ALLISON

(From the Cell Pathology Division, Clinical Research Centre, Harrow, Middlesex, and the WHO World Influenza Centre, National Institute for Medical Research, Mill Hill, London NW7, England)

Cooperation between thymus-derived (T) and bone-marrow derived (B) lymphocytes is required for optimal antibody secretion to thymus-dependent antigens (1–3). However, little is known about which stage of the humoral response is blocked when the helper effect of T cells is lacking. In thymus-deprived animals which do not produce antibody after stimulation by an immunogenic dose of antigen, either antigen recognition, lymphocyte proliferation, synthesis or secretion of antibody might be blocked. Although antibody formation to tobacco mosaic virus (4), bacteriophage (5), and Japanese B encephalitis virus (6) has been reported to be thymus-dependent, little information is available on the role of T- and B-cell cooperation in antibody formation to purified viral antigens, and even less is known about development of immunological memory to such antigens.

We have investigated the role of T-lymphocyte cooperation in the humoral response of mice to purified hemagglutinin (HA)[1] extracted from influenza virus. Our results suggest that the T-cell helper effect is required for the secretion of antibody to HA, but not for the development of specific memory in the B-cell lineage. In the accompanying paper (7) we show that this B-lymphocyte memory can be transferred from one animal to another and can be recalled by heterologous, cross-reacting HA, thus being responsible for the phenomenon of "original antigenic sin."

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‡ Present address: Department of Bacteriology, University of Aberdeen, Forester Hill, Aberdeen, Scotland.

[1] *Abbreviations used in this paper:* CR, cross-reacting population of antibody; HA, purified hemagglutinin; S_1, strain-specific antibodies to H_1 hemagglutinin; So, strain-specific antibodies to Ho hemagglutinin; SRDT, single radial diffusion tests; T^+ mice, immunologically intact mice; TXBM mice, thymectomized, irradiated and bone-marrow reconstituted mice.

Materials and Methods

Viruses. Purified-concentrated preparations of egg-grown Influenza A viruses were obtained as described previously (8) from the following strains: A/BEL/43 (HON1) and A/PR8/34 (HON1) have an HA of subtype Ho; A/FM1/47 (H1N1) has H_1, known to be related to Ho (9, 10); A/Hong-Kong/1/68 (H3N2) has H_3 which is unrelated to Ho and H_1 (11).

Hemagglutinin. In the first set of experiments, Ho was extracted from A/BEL by disruption of the virus with the detergent sodium dodecyl sulphate and separation on cellulose acetate strips (12). In the second set of experiments Ho, H_1, and H_3 were obtained by treatment of the respective virus with the proteolytic enzyme bromelain (13) and separation on sucrose rate centrifugation gradients. Protein estimations of the solutions were performed by the Lowry technique. The biochemical purity of the HA preparations was confirmed by analysis using polyacrymide gel electrophoresis (8). In each case the HA contained two polypeptides (HA_1 and HA_2) the molecular weight of which conformed to those described previously (8). The absence of neuraminidase in the preparations was verified by assay for the enzyme using fetuin. Rabbit hyperimmune sera to our HA preparations were found by hemagglutination-inhibition, neuraminidase-inhibition, and immuno double-diffusion to contain antibody to HA but not to the other surface antigen (neuraminidase) or to the internal antigenic components of the virus (ribonucleoprotein or matrix protein). In the present study mice hyperimmunized with multiple injections of Ho extracted from the egg-grown A/PR8 virus failed to produce antibody to "host" antigen (14). This was demonstrated by the failure of their sera to produce zones on immunoplates containing an egg-grown strain of virus with a different HA.

MICE. Inbred male mice of the CBA strain, 6–8 wk of age were used. To obtain thymus-deprived (TXBM) mice, animals were thymectomized at 6 wk of age, lethally irradiated (900 R) 15–21 days after thymectomy and immediately reconstituted with 6–8 $\times$ 10^6 syngeneic bone marrow cells. Mice found to have thymic remnants at postmortem examination at the end of the experiments were discarded.

IMMUNIZATION. Mice were inoculated intraperitoneally (i.p.) with HA diluted in phosphate-buffered saline, without adjuvant. Sera were obtained from tail bleedings and stored at $-20°C$.

THYMUS CELLS. Thymuses were removed from 6-wk old CBA mice. Single-cell suspensions were obtained by gentle forcing through a stainless steel sieve; they were then washed and resuspended in L15 medium.

MEASUREMENT OF ANTIBODY. This was done by the single-radial-immunodiffusion method (SRDT) (15). Purified-concentrated preparations of influenza virus were mixed in agarose at a final concentration of 0.15 mg of viral proteins per ml of agarose. Antibody potency was measured by the annulus area (mm^2) of the zone of opalescence appearing around 2 mm diameter wells from which 5-μl vol of test antiserum diffused (coefficient of variation of the test = 5%).

ABSORPTION TESTS. This was performed as described previously (16), by mixing equal volumes of antiserum and either a purified-concentrated preparation of influenza virus (5 mg of protein per ml) or saline (dilution control) and testing the mixture in SRDT.

Results

Characterisation of Two Groups of Antigenic Determinants in the HA Molecule and of their Respective Antibody Populations. Potent hyperimmune antisera were raised in rabbits using bromelain-extracted Ho and H_1 antigens and tested in immunodouble-diffusion plates against detergent-disrupted influenza A/PR8, A/FM1, and A/Hong-Kong/68 virus. Fig. 1 and 2 show that, with each antiserum, a precipitin reaction was obtained against both A/PR8 and A/FM1 virus, with typical spurs indicating cross-reactivity. When the anti-Ho serum was absorbed with an equal volume of purified-concentrated A/FM1 virus and ultracentrifuged, the supernatant population of antibody reacted only with

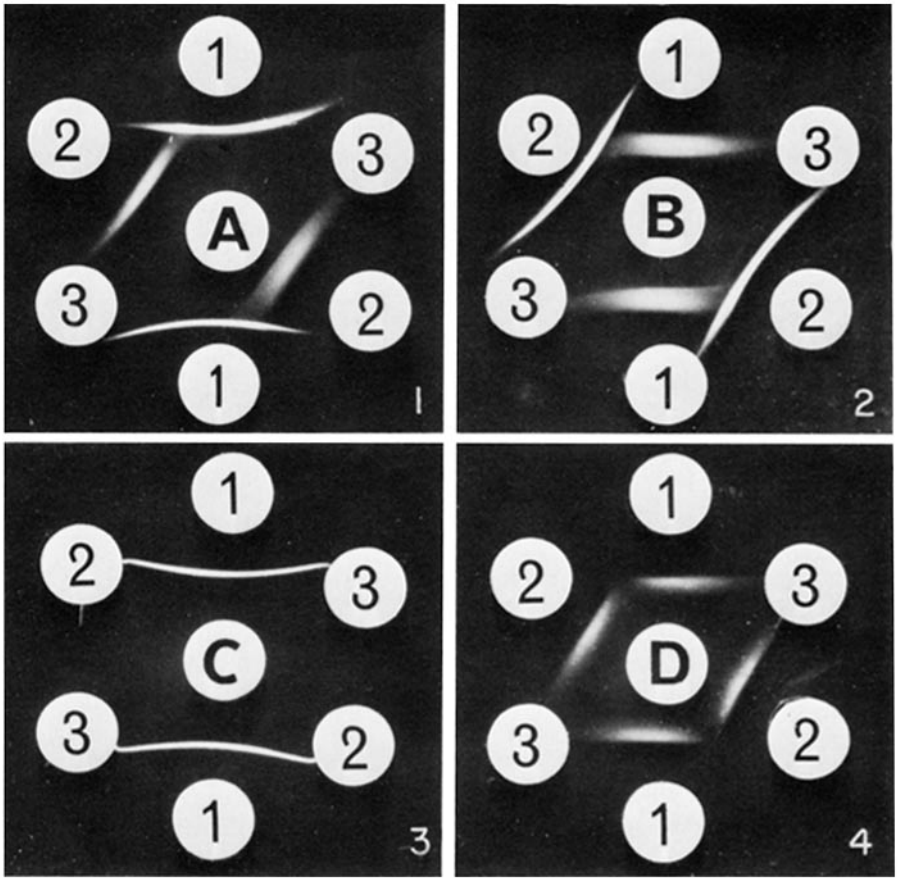

FIG. 1, 2, 3, and 4. Immuno double-diffusion tests. Antigens: to wells marked 1, 2 and 3, 5-μl vol of purified-concentrated A/PR8/34 (HON1), A/FM1/47 (H1N1) and A/HK/1/68 (H3N2) viruses (10 mg virus protein per ml) were added. The virus particles were then disrupted by addition of sodium sarkosyl sulphate (1% final concentration) to each well. Antisera: wells A and B contain antisera prepared in rabbits against bromelain-extracted Ho and H_1 respectively. Well C contains antiserum to Ho after absorption with A/FM1 virus. Well D contains antibody absorbed from anti-Ho serum onto A/FM1 virus and subsequently eluted from the A/FM1 particles. The potencies of the anti Ho and H_1 sera in SRDT are shown in Table I.

A/PR8 HA (Fig. 3). The antibody population eluted from the absorbing A/FM1 virus by low molarity acid buffer reacted with both A/FM1 and A/PR8 HA's (Fig. 4). The unrelated A/Hong-Kong/68 HA did not give any reaction. These results clearly indicate that the immunological system recognizes at least two antigenic determinants (or groups of determinants) within the HA molecule, and responds by making two distinct populations of antibodies. One of them is specific for that part of the HA molecule which is common to Ho and H_1 (cross-reacting population = CR) while the other is strain-specific. We have called the latter population So in the case of an anti-Ho response and S_1 in the case of an anti-H_1 response. Further support for this finding was provided by the results of cross-absorption experiments in SRDT shown in Table I. Anti-HA sera gave large zones of opalescence in immunoplates containing the homologous virus, and smaller zones in plates containing the same amount of heterologous virus. Fig. 5

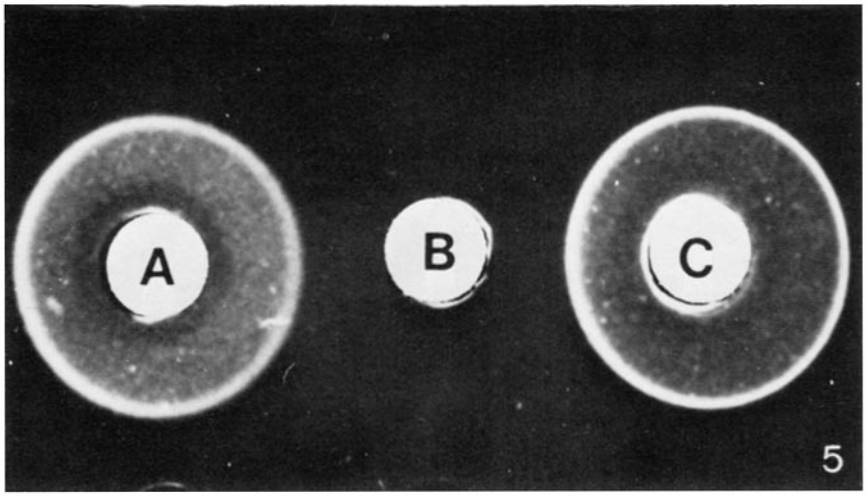

FIG. 5. Absorption test in single-radial diffusion plate: result of a typical experiment. The agarose gel contains 0.15 mg/ml of purified, intact A/PR8/34 virus. Wells A, B, and C contain 5-μl vol of control or absorbed anti-Ho serum (rabbit origin); well A contains anti-Ho serum + saline (control), well B contains anti-Ho serum absorbed with A/PR8 virus, and well C contains anti-Ho serum absorbed with A/FM1 virus.

shows that absorption of an anti-HA serum with the homologous virus is complete, while absorption with the heterologous virus does not reduce the size of the zone. Clearly, two populations of antibodies react in SRDT. The strain-specific population is the more abundant and determines the size of the zone. The CR zone, having a different specificity, lies within but does not interfere with the strain-specific zone. The advantage of cross-absorption tests is that such tests allow an independent quantitation of each antibody population (see Table I). The measurements of antibody in both plates are comparable because each plate contains equal amounts of virus. The potency of the CR population is the area of the zone obtained in a SRDT plate containing the heterologous virus, which is completely absorbed by previous incubation with the homologous virus. The potency of the strain-specific population is the area of the zone obtained in a

TABLE I

Cross-Absorption in SRDT Plates of Anti-HA Sera from Intact Rabbit and Mice or Thymus-Deprived Mice

Serum source	Immunizing antigen	Results on plate containing A/PR8 virus					Results on plate containing A/FM1 virus				
		Control unabsorbed: zone area (mm^2)	Absorbed by A/PR8		Absorbed by A/FM1		Control unabsorbed: zone area (mm^2)	Absorbed by A/PR8		Absorbed by A/FM1	
			Zone area (mm^2)	Redn	Zone area (mm^2)	Redn		Zone area (mm^2)	Redn	Zone area (mm^2)	Redn
				%		%			%		%
Rabbit	Ho	8.8	0	100	8.8§	0	4.2‡	0	100	0	100
Rabbit	H_1	0.7‡	0	100	0	100	7.6	7.6‖	0	0	100
Intact mice*	Ho	8.8	0	100	8.8§	0	2.1‡	0	100	0	100
		3.9	0	100	3.9§	0	0.9‡	0	100	0	100
TXBM mice*	Ho	9.4‡	0	100	0	100	9.5‡	0	100	0	100
		6.5‡	0	100	0	100	6.5‡	0	100	0	100

* Two typical antisera collected 60 days after immunization with three injections of 10 μg of bromelain-extracted Ho.
‡ CR antibody.
§ So antibody.
‖ S_1 antibody.

plate containing the homologous virus, which is not absorbed by the heterologous virus.

Thymus Dependence of Antibody Formation and its Substitution by Repeated Antigen Administration. T^+ and TXBM mice were immunized by graded dilutions of detergent-extracted Ho. Fig. 6 shows a striking difference between T^+ animals, which had a well-defined dose-dependent response even when injected with a 100-fold diluted antigen, and TXBM mice which exhibited no response at all even when injected with the undiluted antigen. Similar results were obtained in experiments in which mice were given two injections of 10 μg of bromelain-extracted Ho (unlisted result). However, this strong thymus dependence of antibody formation could be overcome by a third injection of 10 μg Ho as shown in Fig. 7, but the antibody secretion of TXBM mice was much delayed when compared with the normal humoral response of the controls. When tested in SRDT plates containing A/FM1 virus, these late antisera of TXBM mice gave areas closely similar to those detected in plates containing A/PR8 virus (see Table I), suggesting that CR antibody only was in fact reacting. This interpretation was given further support by the results of cross-absorption experiments shown in Table I. Antibodies from TXBM mice were completely absorbed by A/FM1 virus in a plate containing A/PR8 virus, indicating a complete absence of the So antibody population.

Immunological Reconstitution of Primed Thymus-Deprived Mice. TXBM mice were primed with two injections of 10 μg Ho. No anti-Ho antibodies were detectable in their sera collected 14, 45, and 60 days after the last injection. These primed but nonantibody-secreting mice were reconstituted with thymocytes and boosted with Ho. Table II shows that the antibody response in the CR population was paradoxically lower in reconstituted than in nonreconstituted

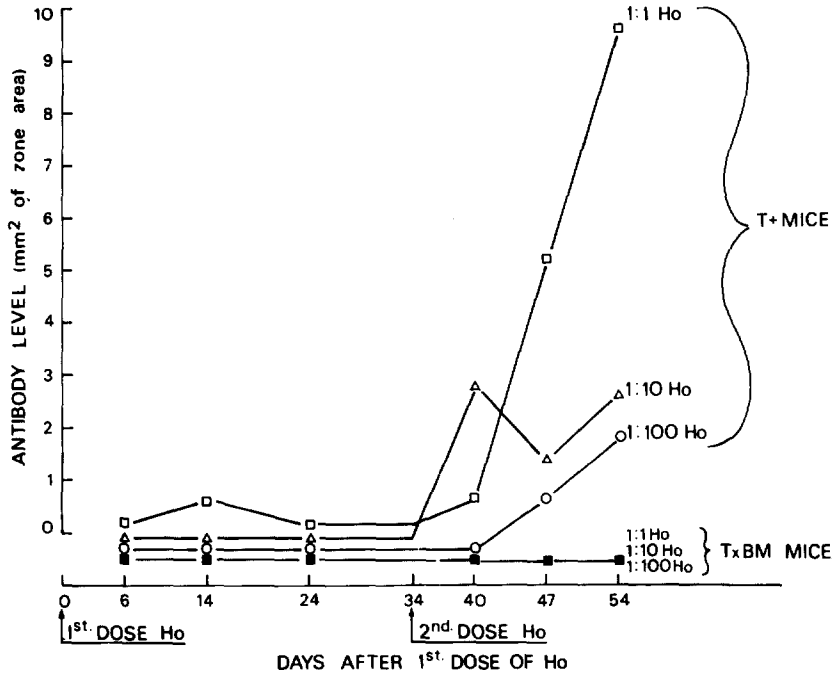

FIG. 6. Anti-Ho antibody responses detected by SRDT of normal (T^+) and thymus-deprived (TXBM) mice after a first and a second injection of antigen. ■, indicates TXBM mice; □, △, ○ indicate T^+ mice immunized with graded dilutions (HA/1 = undiluted, HA/10 = 10^{-1} dilution, HA/100 = 10^{-2} dilution) of a preparation of SDS-extracted Ho containing the equivalent of 50,000 HA U/ml. Antisera were tested individually in single-radial diffusion plate containing A/PR8 virus. Antibody levels are expressed as arithmetic mean of the zone areas for groups of three mice.

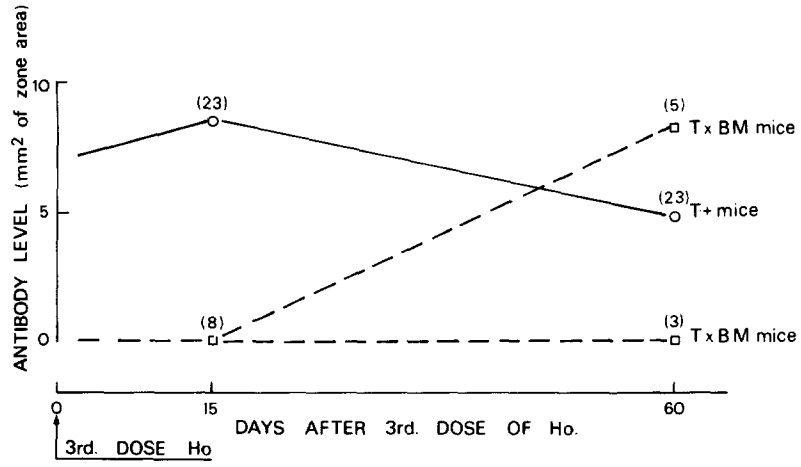

FIG. 7. Anti-Ho antibody responses of normal and TXBM mice after three injections by 10 μg of bromelain-extracted Ho. ○, indicates normal mice (T^+); □, indicates TXBM mice. The number of sera tested is put between brackets. Sera were tested individually in single-radial diffusion tests containing A/PR8 virus; antibody levels are expressed as arithmetic mean of zone areas (mm^2) for each group of mice.

animals. In contrast, a strong, secondary-type So antibody response was observed in reconstituted animals, indicating the existence of immunological memory before reconstitution. Moreover, Fig. 8 shows that memory development was even better in TXBM than in intact animals, since the So antibody response 7, 14, and 21 days after the boost was stronger in primed-reconstituted TXBM mice than in primed T^+ animals.

Discussion

The antibody absorption tests described in this paper, employing either immuno-double-diffusion or single-radial-diffusion to detect antibody, strongly suggest the existence in the HA molecule of two distinct antigenic determinants or groups of determinants. One determinant is cross-reactive for Ho and H_1 hemagglutinins, while the other is apparently strain-specific. This result is in agreement with recent findings by Laver et al. (17) demonstrating the presence of both "common" and specific determinants in the HA extracted from A/Hong-Kong/68, A/England/72, and A/Memphis/72 influenza viruses. Immunologically intact mice or rabbits, immunized with Ho, produced in our experiment two populations of antibodies which correspond to the strain-specific (So antibody) and cross-reactive (CR antibody) antigenic determinants of the molecule. Independent measurement of both populations indicates that the So population of antibodies is more abundant than the CR population, implying that the

TABLE II

Anti-HO Antibody Responses of Primed Thymus-Deprived Mice After Immunological Reconstitution with Thymocytes

Recipient mice	Reconstitution by thymocytes from normal donors‡	Boosting antigens§	Ab response in plate containing A/PR8 virus (So antibody population)			Ab response in plate containing A/FM1 virus (CR antibody population)		
			Mean of zone area		Absorption by A/FM1 virus at day 7	Mean of zone area		Absorption by A/PR8 virus at day 7
			Day 0	Day 7		Day 0	Day 7	
					%			%
TXBM	+	HO	0	0		0	0	
TXBM primed with HO*	+	SRBC	0	0		0	0	
TXBM primed with HO	−	HO	0	2.5 (0–4.4)‖	0	0	12.6 (4.0–35.5)	100
TXBM primed with HO	+	HO	0	10.8 (4.4–25.1)	0	0	2.0 (0–3.9)	100

* Priming consisted in 2 i.p. injections of 10 μg Ho 2 mo before the experiment.

‡ Mice of groups marked + were injected with 1×10^8 syngeneic thymocytes 24 h before the experiment.

§ At day 0 of the experiment, mice received i.p. either 20 μg of Ho or 10×10^6 SRBC.

‖ Range of responses is indicated between brackets.

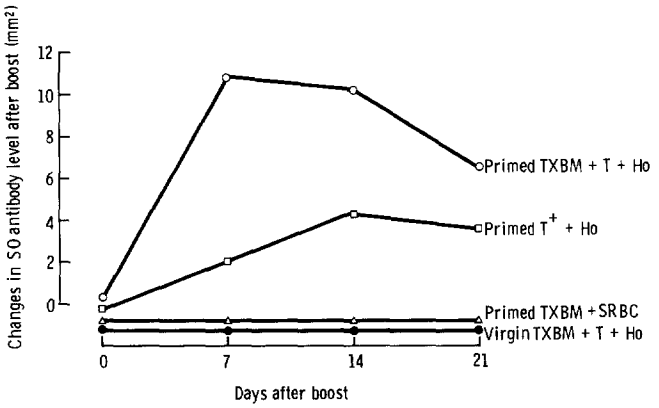

FIG. 8. Increase in So antibody titers during the weeks following a boosting injection in either Ho-primed normal mice (T^+), or Ho-primed thymus-deprived mice subsequently reconstituted (TXBM + T). Techniques of priming, reconstitution by thymocytes, and boosting are described in Table II. So antibody titers were measured in SRDT plates containing A/PR8 virus after absorption of sera by A/FM1 virus, as described in Methods. Changes in antibody titers are expressed as differences (in mm^2 of zone areas) between titers before the boost and 7, 14, and 21 days after the boost.

strain-specific determinant of Ho is more immunogenic than the cross-reactive determinant. However, it is important to emphasize the possible effects on immunogenicity of presenting antigens to the host in different physical states. Bromelain extraction of influenza HA involves both the loss of a portion of the molecule and the production of a soluble protein, as opposed to a particle-bound protein in the case of intact virus. In the present studies, isolated HA was used to induce the immune response while antibody assays were carried out by SRDT tests employing intact virus particles. Such tests would be expected to detect only antibodies capable of reaction with antigens exposed on the virus surface. Knowledge of the relationship of the HA antigenic determinants to the molecular configuration of the hemagglutinin molecule will be of importance, and such studies are currently in progress in our laboratory. It has been suggested that during the "antigenic drift" of influenza viruses, certain determinants of HA are changed while other determinants remain unchanged (16). If, as seems likely, A/FM1 virus evolved in nature from earlier variants including A/PR8 virus, it is striking to observe that their HA's have in common CR determinants which apparently remained stable for 13 yr (interval between A/PR8 and A/FM1 epidemics) while the strain-specific determinants have changed to such an extent that these viruses have been classified in two different groups on the basis of their failure to cross-react in hemagglutination-inhibition tests.

The results described in this paper demonstrate a strong thymus dependence of antibody formation against HA. However, the refractoriness of TXBM animals to produce antibody can be overcome by repeating the injections of antigen: under such hyperimmunizing conditions some TXBM mice do produce antibody but this production seems qualitatively incomplete: only the population of CR antibodies is secreted. This result indicates a different handling of antigenic determinants on the molecule by the immunological

system. The strain-specific determinant of the molecule behaves as a highly thymus-dependent antigen, while the cross-reactive determinant behaves as a relatively thymus-independent antigen. These observations are not without precedent: Senyk et al. (18) demonstrated that some determinants of the glucagon molecule are recognized by T cells, while others are recognized by B cells only. We believe that the immune response to influenza hemagglutinin provides another example of functional dissection of a molecule by the immunological system.

The kinetics of antibody formation in TXBM mice appear to be very unusual: antibodies are not detected at the time when normal control animals exhibit their maximal response. In contrast, 2 mo after priming, high levels of CR antibodies are detectable in TXBM mice, while the antibody level of controls is already low. Since the CR determinant of HA is normally a weak immunogen, the CR antibody response of TXBM mice must be considered both as unusually delayed and unusually abundant. This curious phenomenon suggests that some mechanism exerting feedback regulation of the humoral response is lacking in thymus-deprived animals. Two suppressive factors of the humoral response have been described: one is IgG antibody itself (19, 20) and the other is a population of suppressor T cells (2). Production of CR antibodies is much greater in nonreconstituted primed TXBM mice than in reconstituted animals. This suggests that the injected thymocytes suppressed the CR antibody response, thus behaving as suppressor T cells. Further evidence for a regulatory influence by T cells in our system is given in the accompanying paper.

Our observation that specific "memory cells" are generated by HA in TXBM mice in the absence of detectable secretion of anti-HA antibody suggests that development of immunological memory in B cells is a thymus-independent process, even for thymus-dependent antigens. A similar interpretation has been given by Roelants and Askonas (21) to their observations on antibody formation against *Maia squinado* hemocyanin in thymus-deprived mice. It is widely assumed that the generation of "memory cells" involves antigen recognition and induced proliferation of specific clones of lymphocytes to form more cells recognizing the same antigen (22). On the hypothesis of monoclonal expansion, which on grounds of economy seems likely, our results suggest that the T-cell helper effects are exerted on a late stage of the humoral response, namely the differentiation of already proliferating specific B cells into antibody-secreting cells.

Moreover, the antibody response of primed TXBM mice after reconstitution with thymocytes was striking in its rapidity and magnitude, suggesting that there is an accumulation of memory cells in such animals, despite the deprivation of T-helper cells. It could be argued that a few T lymphocytes may remain in TXBM mice (23) and may provide some help to the proliferation of B cells. However, these animals failed to produce any detectable antibody response to an immunogenic dose of antigen, which implies an absence of demonstrable T-helper effect. If a T-cell helper effect is required for B-cell proliferation, it would be surprising that such animals would develop B memory of normal magnitude, and still less likely that the memory should be greater than in intact mice. Indeed, such an accumulation of memory cells in TXBM animals suggests

that T cells may exert a negative effect on memory development in normal animals. After a "proliferation signal" provided by antigenic stimulation, precursor B cells appear to proliferate independently of T cells, as demonstrated in vitro by Askonas et al. (24). This thymus-independent process would result in an antigenically specific pool of B cells which have memory function but as yet no antibody-producing capability. The role of T cells during a normal humoral response could be to provide a "differentiation signal" enabling these memory cells to differentiate into antibody-secreting cells. In doing so, T cells could divert some progeny of each expanding B clone into antibody-secreting, nonproliferating cells which are in this way removed from the population of specific memory cells. This diversion by T cells could be an essential regulatory mechanism during the humoral response to thymus-dependent antigens. The timing and the intensity of the diversion could decide the balance between the development of B-memory cells and antibody-forming cells.

Summary

Using immunodiffusion methods it has been shown that purified hemagglutinin (HA) extracted from two related strains of influenza A viruses (A/PR8/34 and A/FM1/47) have two distinct antigenic determinants, or groups of determinants. One determinant is cross-reactive while the other is strain-specific. Antisera raised in normal mice against HA were shown to contain two populations of antibody molecules, each directed against one of the determinants. Immunization of thymus-deprived (TXBM) mice showed a strong thymus dependence of antibody formation to HA. However, the thymus dependence of antibody formation against the cross-reactive determinant could be overcome by repeated inoculations of HA in TXBM mice, indicating a different handling of two portions of the same molecule by the immunological system. Strong, secondary-type responses to the strain-specific determinant were observed in primed thymus-deprived mice after reconstitution with virgin thymus cells, showing that specific immunological memory was elicited by this determinant despite the absence of detectable antibody secretion. These findings are interpreted as examples of immunological recognition and memory mediated by B lymphocytes and discussed in terms of mechanisms of T and B lymphocyte co-operation. It is suggested that the helper effect of T lymphocytes is exerted at a late stage in the differentiation of specific populations of B cells into antibody-secreting cells.

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