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ROLE OF THE THYMUS IN TOLERANCE. VII. DISTRIBUTION OF NONAGGREGATED AND HEAT-AGGREGATED BOVINE GAMMA GLOBULIN IN LYMPHOID ORGANS OF NORMAL NEWBORN AND ADULT RATS‡

The study reported here forms an essential part of our investigations of the effect of antigen, given either systemically or directly into one of the lymphoid organs, in the induction of specific immunologic tolerance.[1-5] For both soluble protein and homograft systems, it has been established that tolerance induced by neonatal administration of antigen can be transferred in later life by grafting of the thymus to suitable recipients. In normal adults, deprived of a peripheral lymphocyte pool by irradiation, similar tolerance is readily induced by intrathymic injection of antigen, much smaller amounts being effective when given by this route than systemically. In the presence of a peripheral lymphocyte pool, tolerance for several immune responses shows a striking increase over several days after intrathymic injection and at most a slight increase with systemic antigen. Finally aggregated antigen is as effective as soluble material in inducing tolerance, when injected directly into the thymus, but much less effective by other routes. The thymus thus appears unique in its relation to tolerance, since similar manipulations of other lymphoid organs (lymph nodes, spleen) fail to produce tolerance greater than that obtained with manipulation of the peripheral pool itself.

These findings are consistent with the now well-established fact that the thymus is the source of a population of lymphocytes which enter the peripheral pool and act as precursor cells for several types of immune response.[6,7] A number of questions are implied which have received partial answers in recently published work. One concerns possible quantitative or qualitative differences between thymus and peripheral lymphocytes in their

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reactivity to antigen. Tolerance can be produced by suitable antigens in thymectomized adults,[8,9] *i.e.* by direct action on peripheral cells, but an exact comparison of the reactivities of the two cell types (peripheral lymphocytes and thymus lymphocytes) has not been achieved. A second problem concerns the blood-thymus barrier and its role in excluding particulate antigens in particular from the thymus, where they might most effectively induce tolerance. It is well established that a barrier exists in adults but is relatively slight in the perinatal period.[10-18] Finally, the absence in the thymus of an antigen-trapping mechanism, which plays the dual role in other lymphoid organs of clearance and of antigen-processing essential to the immune response, may be of major importance in permitting antigens which gain access to the thymus to induce tolerance over an extended period of time. An elegant recent study by Mitchell and Nossal has established the relevance of these last two points and provided comparative data for several antigens differing in character and state of aggregation.[12,13] In the present paper, we provide additional data obtained in normal newborn and adult rats with "soluble" and heat-aggregated bovine γ-globulin (BγG), the antigens used in our recent tolerance studies. The findings confirm and extend the findings of Mitchell and Nossal.

MATERIALS AND METHODS

Experimental animals

Lewis and Sprague-Dawley rats were obtained from Microbiological Associates, Bethesda, Md., and Charles River Laboratories, Brookline, Mass., respectively. They were used within two hours after birth or at 4-5 or 8-10 weeks of age.

Antigens

BγG (lot #A30702, Armour Pharmaceutical Laboratory, Kankakee, Ill.) was processed as follows to give soluble and aggregated preparations:[4,5] A 2% BγG solution in saline (8 ml.) was heated at 62-64°C. with gentle shaking for 18 minutes, cooled in ice for 30 minutes, and spun in a Spinco model L-1 ultracentrifuge with a #40 rotor at 40,000 r.p.m. (105,536 *g* at the middle of the tube) for 120 minutes. The upper third of the supernatant represented the "soluble" fraction. The pellet was homogenized in 10 ml. saline with a glass tissue homogenizer, kept at 4°C., and centrifuged and resuspended in saline twice daily for 6-7 days. In this time, the amount of protein in the pellet diminished to approximately 60% of the starting value. The later washes contained small but measurable amounts of protein (< 10 μg/ml.). The final homogenous suspension was the "aggregated" preparation. In each experiment, only freshly prepared soluble and aggregated materials were employed.

Labelling

BγG was labelled with I^{125} (obtained as carrier-free Na I^{125} from New England Nuclear Corp., Boston, Mass.) with the use of the chloramin T technique.[14] Iodinated

BγG (4 ml., 2,000 μc) was added to 8 ml. of 2% crude BγG solution, which was processed as described above. Label was distributed between soluble and aggregated fractions in proportion to the amount of protein in each. The specific activity in different experiments varied between 0.4 and 88 μc/mg. protein. At the end of the washing procedure, the total label remaining in the aggregated protein preparation was about 60% the starting value. Radioactivity in the final washes was always less than 5×10^4 c.p.m. per ml.

Distribution studies

Labelled crude, soluble, or aggregated BγG were injected intravenously or intraperitoneally, the dose being adjusted in terms of the size of the animal and duration of the experiment. Adults received 70, 90, and 200 μc of I^{125} BγG and newborns 2, 3, and 50 μc respectively. Control rats were injected with NaI^{125}. Two or more rats in each group were sacrificed 1, 7, and 30 days after the injection and exhaustively perfused through the heart with warm saline (about 50 ml. in newborn, 300 ml. in adult). A blood sample was taken by cardiac puncture before perfusion, and the thymus, cervical lymph nodes, spleen, liver, lungs, kidneys, and heart were removed immediately afterward. Individual organs were weighed and counted in a well-type scintillation counter (Baird Atomic single channel γ-spectrometer, model 530). The total amount of label in each organ was expressed, after corrections for background and decay, as per cent of the total injected dose. An alternative calculation expressed the corrected c.p.m. per gram in the organ as a percentage of the c.p.m. per gram total body weight originally injected.

The distribution of labelled antigen within the thymus, lymph nodes, and spleen was investigated by teasing each organ in cold Hanks' solution, allowing the "stroma" to settle by gravity for five minutes in a centrifuge tube, separating "cells" from stroma, and washing each four times with fresh Hanks', the radioactivity of pellet and supernatant being counted after each centrifugation.

Autoradiograms were prepared from paraffin sections, as in earlier studies, with the use of Kodak AR-10 stripping film. They were exposed 4-6 weeks, developed in Kodak D-19, and stained with Giemsa.

Identification of BγG in thymus

Perfused thymus glands, removed 1 or 7 days after intravenous injection of labelled BγG (or of NaI^{125}), were homogenized in saline and subjected to sonication in an MSE ultrasonic disintegrator, Model 60 W, for 90 seconds. Radioactivity was counted in the supernatant and pellet obtained by centrifugation at 20,000 r.p.m. for 60 minutes (about 30,000 *g*). The pellet was washed twice with saline and centrifuged again; the combined washings, containing about 10% of the radioactivity of the pellet, were treated as a second supernatant. Each supernatant was added to an antibody excess mixture containing 100 μg BγG and 1 ml. of hyperimmune rabbit antiserum (approximately 2 mg. of specific antibody against BγG). The mixture was incubated at 37°C. for 120 minutes and centrifuged 10 minutes at 1,500 r.p.m. The amount of biologically active antigen in the thymus sonicate was calculated from the radioactivity of the antigen-antibody precipitates obtained. The second supernatant always contained less than 2% of the label present in the first.

A sonicate of perfused thymus, prepared 24 hours or 7 days after intravenous injection of 150 μc I^{125}-BγG, was centrifuged at 2,000 r.p.m. for 10 minutes and the super-

natant tested for antigen by gel diffusion autoradiography.[15] The test extract was allowed to diffuse for 20 hours against hyperimmune rabbit anti-BγG in a microslide system. The slide was then washed over 12 hours in several changes of saline, dried in contact with filter paper at 37°C., and stained with 1% Nigrosin in 2% acetic acid for 5 minutes. Film (Kodak blue brand medical X-ray film) was placed and held firmly between the dry, stained slide and a clean slide and wrapped in parafilm "M." The film was developed after four weeks of exposure. Specificity controls were not included.

RESULTS

Quantitative distribution of injected antigen in various lymphoid organs

Raw data, obtained by counting perfused organs of adult rats given labelled soluble or aggregated BγG, are shown in Table 1. Average values for the concentration of antigen in each tissue, relative to the total injected, are also presented graphically in Figure 1. Concentration presumably determines the frequency of contact between antigen and susceptible lymphocytes, and may therefore be the more relevant figure in a discussion of the mechanism of tolerance. The total value for a given organ, on the other hand, merely expresses its size.

The data are seen to be fairly reproducible, even in rats of different strains. Soluble BγG, by 24 hours, penetrated all organs examined to a more or less equal extent, the lung showing a somewhat higher concentration of label than other organs. Penetration into the thymus was only slightly less than into spleen or lymph node and effectively as great as penetration of liver, kidney, etc. The concentration of label fell uniformly over 30 days in all organs, more or less in parallel with the fall in blood level. The kidney value, however, remained high, possibly because there was continuing excretion of labelled material (antigen or its breakdown products). These data demonstrate passive spread of soluble antigen throughout the organism with little distinction among organs.

Aggregated antigen was taken up in liver and spleen (and to a lesser extent lung) in large amounts over the first 24 hours, presumably because of the reticuloendothelial function of these organs. Conversely, in the thymus and lymph node, as well as heart, less than one tenth as much antigen penetrated as in the case of soluble material, whether because of rapid clearance and the low level of antigen in the circulation or because of the blood-tissue barrier in each case. The progressive fall in blood concentration was paralleled by a decreasing content of label in the thymus, heart, etc. However, in spleen, lymph node, and lung the concentration remained high relative to concentrations in other organs and in animals

TABLE 1. DISTRIBUTION OF LABELLED ANTIGEN IN LYMPHOID AND OTHER ORGANS OF ADULT RATS

Organ	Average weight (gm.)	Label found at various intervals after intravenous injection*						
		Soluble BγG			Aggregated BγG			$Na\ I^{125}$
		1 day	7 days	30 days	1 day	7 days	30 days	1 day
Blood	28.0	18.4(278) 20.9(315) 22.4(290)L	4.3(64) 2.6(39)	.033(.51) .033(.51)	.59(8.9) .49(7.4)	.054(.82) .026(.38)	.0039(.058) .0044(.064)	.67(10)L .50(7.5)L
Thymus	.48	.034(21) .027(16) .017(14)L	.004(4.8) .008(4.9)	.0008(.09) .0001(.12)	.002(1.1) .002(1.5)	.0003(.29) .0002(.13)	.00005(.08) .00004(.04)	.0035(3.9)L .0022(2.4)L
Cervical nodes	.81	—(45) —(45) —(86)L	—(21) —(5.5)	—(.22) —(.26)	—(4.7) —(6.5)	—(1.7) —(1.3)	—(6.8) —(.39)	.0016(2.9)L .0015(3.1)L
Spleen	.91	.057(24) .075(36) .034(17)L	.012(6.5) .013(5.1)	.0004(.24) .0004(.20)	.17(56) .29(85)	.046(24) .059(17)	.0048(2.6) .0019(1.9)	.0051(3.0)L .0038(2.3)L
Liver	18.0	.63(13) .59(13) .41(8.2)L	.047(1.0) .064(1.1)	.0029(.07) .0034(.09)	.57(12) 1.14(24)	.22(4.5) .23(4.8)	.007(.17) .012(.31)	.24(6.1)L .21(5.5)L
Lung	3.2	.75(100) .27(35) 1.61(119)L	.078(10) .034(4.6)	.0016(.33) .0019(.39)	.05(6.7) .12(17)	.002(.21) .003(.40)	.007(1.4) .008(1.7)	.022(1.6)L .028(1.7)L
Kidneys	3.8	.17(17) .13(14) .18(15)L	.027(2.9) .033(2.7)	.0059(.61) .0040(.59)	.10(11) .11(11)	.037(4.6) .048(5.4)	.0048(.66) .0052(.68)	.035(3.8)L .032(3.8)L
Heart	1.6	.099(25) .078(19) .17(29)L	.017(4.8) .009(2.5)	.00034(.15) .00027(.10)	.003(0.9) .005(1.5)	.0004(.12) .0003(.09)	.0001(.031) .0009(.029)	.0037(.90)L .0029(.86)L

* Duplicate determinations in different adult male, Sprague-Dawley rats or Lewis rats (marked L), injected intravenously with soluble or aggregated BγG or with Na I^{125}. Values represent percentage of injected I^{125} found in whole organs and (in parentheses) cpm/gm as per cent of cpm/gm whole body weight injected.

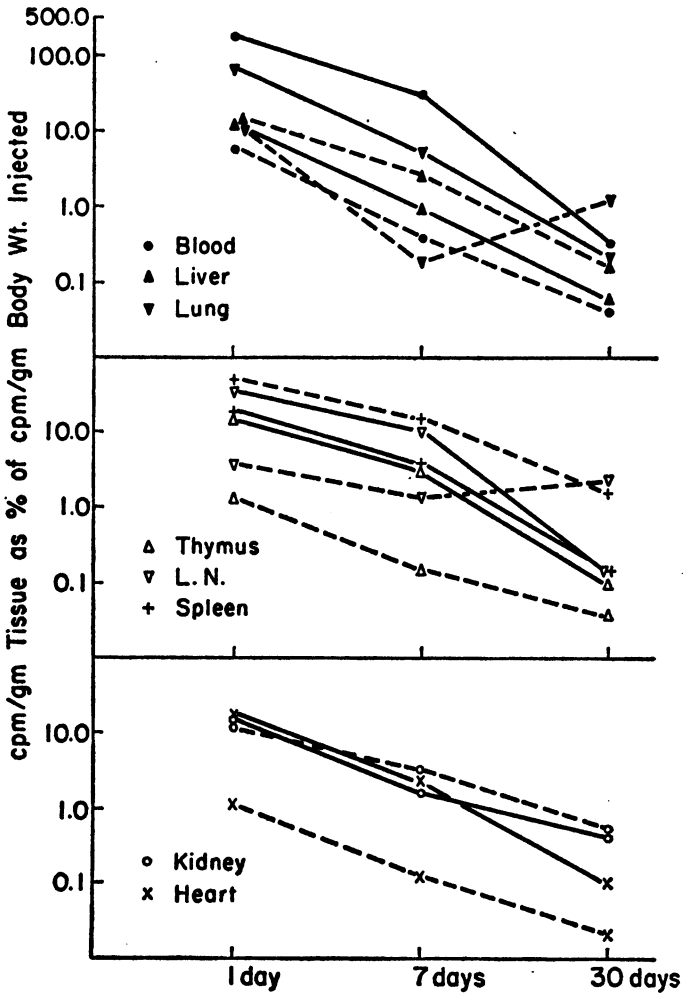

FIG. 1. Relative concentrations of injected I^{125}-BγG in blood and various organs of adult rats at intervals following intravenous injection (averages of data given in Table 1). Solid lines represent values obtained with soluble BγG and dashed lines values with aggregated BγG.

given soluble BγG. The mechanism here is unknown. The kidney value remained high throughout, again probably reflecting excretion of breakdown products of phagocytized BγG.

Similar data obtained in newborn Sprague-Dawley rats are presented in Table 2 and Figure 2. Lymph node values obtained at 24 hours were regarded as unreliable and are not given here. The unusually high concen-

TABLE 2. DISTRIBUTION OF LABELLED ANTIGEN IN LYMPHOID AND OTHER ORGANS OF NEWBORN RATS

	Average weight (gm.)			*Label found at various intervals after intraperitoneal injection**					
				Soluble BγG			*Aggregated BγG*		
Organ	*1 day*	*7 days*	*30 days*	*1 day*	*7 days*	*30 days*	*1 day*	*7 days*	*30 days*
Blood	.34	1.1	5.5	12.6(222) 11.4(200)	4.97(72) 4.36(63)	.38(5.6) .37(5.5)	1.85(32) 3.08(54)	1.06(15) 0.96(13)	.018(.26) .018(.26)
Thymus	.013	.041	.31	.041(24) .031(18)	.006(3.4) .012(3.9)	.0010(.35) .0017(.40)	.013(5.0) .015(4.5)	.023(10) .013(4.0)	.0002(.057) .0003(.058)
Cervical node	—	.002	.04	— —	—(48) —(65)	—(.95) —(.79)	— —	—(39) —(28)	—(.30) —(.20)
Spleen	.015	.15	.33	.055(22) .038(15)	.038(4.0) .052(5.6)	.0021(.55) .0025(.62)	.024(14) .033(13)	.032(3.5) .037(4.0)	.00073(.15) .00050(.14)
Liver	.25	.49	3.0	.89(20) .91(21)	.049(1.7) .098(2.8)	.015(.42) .015(.42)	.37(9.3) .44(9.9)	.076(2.9) .037(1.1)	.0064(.16) .0065(.17)
Lung	.17	.56	2.0	.28(8.8) .25(7.1)	.035(1.1) .046(1.2)	.0069(.29) .0068(.28)	.19(7.6) .15(6.8)	.027(.82) .028(.73)	.0016(.062) .0015(.058)
Kidney (bilateral)	.06	.23	.83	.40(48) .54(41)	.089(6.5) .149(9.6)	.010(.89) .008(.62)	.16(15) .18(17)	.23(16) .12(9.6)	.0039(.36) .0085(.74)
Heart	.03	.12	.41	.098(19) .109(21)	.019(2.6) .012(1.7)	.0018(.36) .0016(.29)	.028(5.7) .032(5.5)	.013(1.7) .010(1.3)	.00025(.052) .00033(.063)

* Sprague-Dawley rats. Data presented as in Table 1.

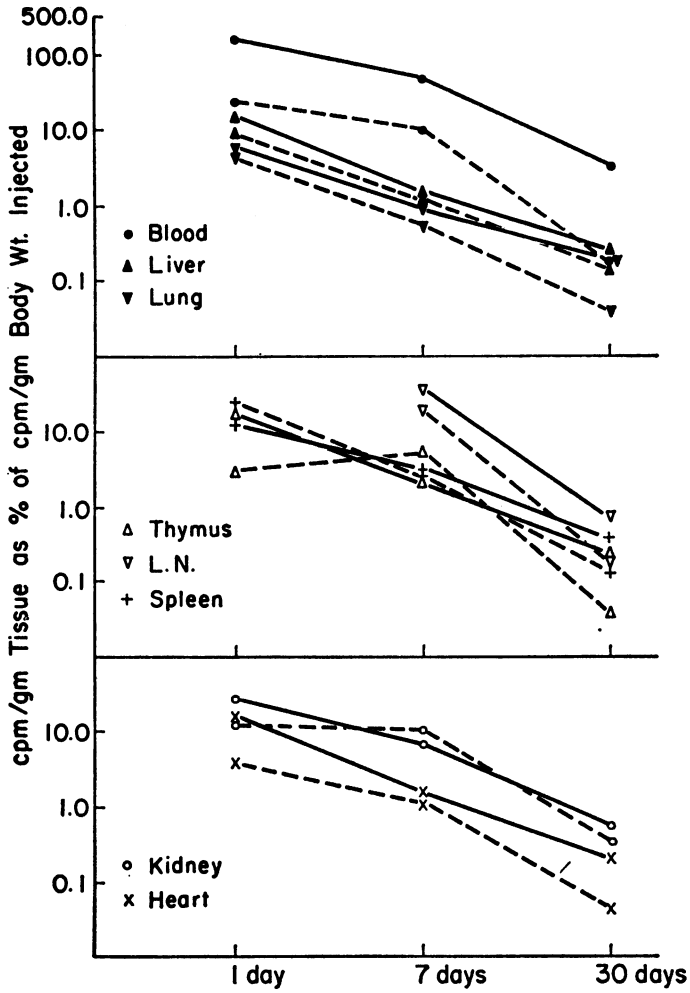

FIG. 2. Relative concentration of injected I^{125}-BγG in blood and various organs of newborn rats at intervals following intraperitoneal injection (averages of data given in Table 2). Data plotted as in Fig. 1.

trations recorded in the nodes at 7 days were thought, on the basis of autoradiographic evidence, to depend on pooling of antigen in the sinuses, where it was not removed by the perfusion procedure. As in adult rats, soluble BγG gave a picture of uniform, passive diffusion; all organs showed a similar concentration of label, roughly a constant proportion of the blood level, and a slow, uniform decline. Again the kidney value remained high. Aggregated antigen gave values only slightly lower than soluble except in

the reticuloendothelial organs. Label actually increased in the thymus between 1 and 7 days, suggesting continuing slow penetration of the thymus by circulating antigen. Here the pattern was strikingly different from that in adults. These findings, however, may have been determined as much by the deficient clearance mechanism of the newborn and consequent persistence of circulating antigen as by a lesser blood-thymus barrier.

Tables 3 and 4 show the result of a simple fractionation study, in which the amount of label was compared in "cells" (almost entirely thymocytes), "stroma" (including phagocytic and epithelial reticulum cells and some thymocytes), and the intercellular fluid. One and 7 days after injection of soluble antigen in adult rats, a very small proportion of the label present in thymus and lymph node was actually in the stroma, presumably within phagocytic cells, and only about 1% in the thymocytes. Almost 90% was extracellular. In the spleen, several times more label was found in the stroma. By 30 days, there was a relative increase in stromal and cellular label in all organs examined. Well over half, however, remained extracellular in the thymus and nodes. Aggregated BγG, promptly after injection, entered the stroma in much larger amount than soluble BγG in spleen and nodes. In the thymus, a good deal was present in the cell fraction at 24

TABLE 3. DISTRIBUTION OF LABEL WITHIN DIFFERENT LYMPHOID ORGANS IN ADULT

Anatomic element	*Percentage of total organ label in different anatomic elements**					
	Soluble BγG			*Aggregated BγG*		
	1 day	*7 days*	*30 days*	*1 day*	*7 days*	*30 days*
Thymus						
Stroma	11.3	6.7	31.8	9.9	70.5	26.5
	13.9	6.9	21.7	10.9	21.7	11.0
Cells	1.3	0.8	7.6	8.9	9.7	7.6
	1.5	0.5	13.0	8.3	4.2	14.0
Cervical node						
Stroma	8.6	9.0	19.8	24.1	17.9	3.0
	9.7	15.0	28.8	24.7	38.5	39.5
Cells	0.5	0.4	0	3.9	15.3	3.6
	0.4	1.0	14.4	6.0	20.3	9.1
Spleen						
Stroma	21.4	21.2	66.5	59.5	46.6	67.6
	28.7	25.3	54.9	47.2	52.3	63.7
Cells	1.2	1.5	6.6	13.5	17.2	23.0
	1.2	3.1	6.2	15.3	13.2	20.9

* Duplicate determinations in adult male, Sprague-Dawley rats given labelled antigen intravenously.

TABLE 4. DISTRIBUTION OF LABEL WITHIN DIFFERENT LYMPHOID ORGANS IN NEWBORN

Anatomic element	Percentage of total organ label in different anatomic elements*					
	Soluble BγG			Aggregated BγG		
	1 day	7 days	30 days	1 day	7 days	30 days
Thymus						
Stroma	4.9	9.5	18.9	16.8	14.9	54.5
	6.1	11.0	20.3	14.9	16.9	53.0
Cells	0	0	3.8	0	0	9.1
	0	0	2.4	0	0	10.4
Cervical node						
Stroma			8.9			25.9
			13.7			24.5
Cells			6.3			22.2
			5.7			20.4
Spleen						
Stroma	16.3	13.9	38.9	37.4	27.2	38.2
	22.9	34.5	35.5	30.9	23.1	57.3
Cells	0	0	3.3	0	2.1	5.5
	0	0.78	2.4	0	2.0	3.1

* Duplicate determination in newborn Sprague-Dawley rats given labelled antigen intraperitoneally.

hours and more in stroma at 7 days. The 30 day values were highly variable. In newborn animals more label remained extracellular in all organs than in adults. The thymus cells did not contain measurable label at 1 and 7 days after either soluble or aggregated material. In the spleen similarly, no label was found in the cell fraction at 1 day, with either material, and little or none even 7 days after soluble BγG.

Data obtained in Lewis rats of various ages injected with "crude" (uncentrifuged) BγG are presented in Table 5. These are considered of interest, since the usual form in which antigen is presented to the organism includes both soluble and aggregated elements. In the adult, little difference was apparent between animals given antigen intravenously and intraperitoneally. There was extensive uptake in reticuloendothelial organs (liver, spleen, lung) at 24 hours, like that seen with aggregated BγG. In the blood and the thymus, as well as heart and kidney, the level of uptake reflected uptake of the soluble component. Newborn rats showed a pattern of penetration of antigen into various organs much like that seen with soluble material in newborn Sprague-Dawley animals. Antigen uptake in 4-5 week old animals resembled that in adults more than in newborns.

TABLE 5. DISTRIBUTION OF LABELLED CRUDE BγG IN LEWIS RATS OF VARIOUS AGES

	Label found at various intervals after injection							
	Newborn rats (ip)			*4-5 week rats (ip)*		*Adult rats (ip)* or (iv)*		
Organ	*1 day*	*7 days*	*28 days*	*1 day*	*5-7 days*	*1 day*	*6-7 days*	*27 days*
Blood	—	11.5(64)	.64(8.9)	23.5(351)	4.77(67)	36.6(510)	5.30(71)	.037(.60)
	2.3(40)			18.4(269)	5.96(58)	27.2(381)*	4.85(65)	.025(.47)
						25.9(400)*	2.86(36)*	
Thymus	.055(24)	.003(4.1)	.0028(.57)	.26(51)	.018(5.1)	.037(33)	.010(9.3)	.00013(.17)
	.12(12)			.11(27)	.017(3.4)	.0054(57)*	.009(7.3)	.00004(.59)
						.015(17)*	.007(6.3)*	
Cervical	—	—(14)	—(4.3)	—	—	—	—	—(.12)
nodes	—(31)			—(64)	—(12.5)	—(109)*	—	—
						—	—(11)*	
Spleen	.17(30)	.14(5.2)	.0064(1.2)	.23(42)	.017(3.8)	.11(62)	.016(9.5)	.00072(.47)
	.042(9.9)			.19(45)	.024(4.8)	.12(66)*	.012(6.0)	.00060(.39)
						.092(57)*	.006(3.4)*	
Liver	1.19(27)	.48(5.4)	.074(1.4)	2.33(47)	.15(2.9)	2.50(54)	.42(8.7)	.0073(.18)
	—			—	.48(10)	—	.34(7.0)	.0045(.12)
						1.05(26)*	.13(2.9)*	
Lung	.71(27)	.75(7.4)	—	3.93(118)	.40(12)	2.05(165)	.23(18)	.0043(.41)
	—			—	.26(8.5)	—	.072(5.5)	.0032(.29)
						—	.061(5.1)*	
Kidneys	.42(37)	.51(11)	.039(28)	1.31(95)	.11(7.8)	1.08(101)	—	.0046(.51)
	—			—	.058(4.4)	—	—	.0039(.42)
						—	.047(4.1)*	
Heart	—	.12(5.4)	.011(1.5)	—	.05(7.8)	.27(50)	.057(10)	.00057(.13)
	—			—	.03(4.3)	—	.031(5.6)	.00041(.09)
						—	.024(4.3)*	

* Adult rats injected intraperitoneally (ip).
Data presented as in Table 1.

Distribution of labelled antigen within lymphoid organs

Autoradiography was used to obtain a more precise localization of antigen within individual lymphoid organs. Following injection of labelled crude BγG intraperitoneally in newborn rats, label was diffusely present, both intra- and extracellularly throughout the thymus and lymph nodes by 24 hours. The concentration, as judged by the number of silver grains was highest in the walls of blood vessels and in the immediately adjacent parenchyma. In the spleen, it was found mostly in and about the vessel walls and throughout the white pulp and marginal zone; little was present in the red pulp. With soluble BγG, the distribution after 24-36 hours was precisely the same. With aggregated BγG, the distribution was similar but there was a sharper decrease in concentration of label with distance from the vessels and several areas of parenchyma, in each of the three organs examined, appeared to contain none.

Seven days following the injection of labelled antigen, there was little change in the distribution observed. In the thymus, extracellular label continued to predominate, especially in the vicnity of vessels. More label was present in the medulla than the cortex. There was a distinct increase in labelled material, in the vessel walls themselves and the interlobular septa, mainly within phagocytic cells. In both lymph nodes and spleen, antigen was also diffusely present, though most concentrated in vessel walls and the neighboring parenchyma. Extracellular antigen predominated near vessels; however, label was also seen within a variety of cells, with the single exception of cells resembling blasts. By 28 days, a few grains, representing label and presumably antigen, could still be identified in all three organs. In the thymus, these were found in scattered areas of the medulla, especially near vessels.

In 5-week old rats, label within the three organs showed an essentially adult pattern of distribution one day after intravenous injection of crude I^{125} BγG. Between 1 and 7 days, there was a more rapid decrease of extracellular antigen in the thymus parenchyma than in the newborn rats.

In adult rats, soluble or crude labelled BγG, injected systemically, penetrated in substantial amounts to the parenchyma of all three lymphoid organs studied within 24-36 hours, though much less than in the newborn. Where label was present, it appeared both intra- and extracellular, and was seen in both nuclei and cytoplasm of small and large cells. In the thymus, more was present in the medulla than the cortex, more in the parenchyma near vessels than at remote sites, and much more in the walls of vessels themselves (and in the accompanying lymphatics) than in the adjacent parenchyma. In lymph nodes and splenic white pulp, there was a very low level of diffuse label, clearly distinguishable from background, mostly near

vessels and in vessel walls. Little or none was present in the splenic red pulp. There was no preferential localization in the germinal centers or marginal zone or in the lymph node medulla with soluble antigen, though substantial amounts were present in the (unperfused) lymph node sinuses. With crude and with aggregated BγG, however, there was a high concentration of the injected material at these three sites, as noted by a number of authors, and little of the aggregated material was found in the parenchyma.

By 7 days after crude antigen, there was a marked decrease in label within the thymus, and most areas of thymus cortex contained none. The residual label was mainly extracellular in scattered, patchy areas of the medulla. In the lymph node, label was still present at low levels throughout the cortex and the lymphocytic follicles, especially in vessel walls; it was now concentrated to a significant degree in germinal centers, and was also present in medullary sinuses, perhaps as a stagnant pool.

A number of illustrative autoradiographs are shown in Figure 3.

Identification of biologically active BγG in thymus

Of the I^{125} found in the thymus of adult rats 1 or 7 days after intravenous injection of labelled crude BγG, approximately 70% could be extracted by a single brief sonication (Table 6). Of this extracted label 70-80% (roughly half the I^{125} in the thymus) was coprecipitable with BγG and specific antibody, and could be presumed to be BγG or an antigenically intact fragment of BγG. The ability of this material to give a precipitation

TABLE 6. QUANTITATIVE COPRECIPITATION OF I^{125}-LABELLED ANTIGEN IN THYMUS OF ADULT RATS

Injected BγG			*Radioactive label (cpm) found***				
				Sonicate			
Protein (μg)	*Label (μc)*	*Day of sacrifice*	*Whole organ*	*Pellet*	*Supernatant*	*Precipitated in immune complex*	*Not precipitated*
450	40	1	13,436	3,933	8,457	5,817	2,345
			11,662	3,531	7,836	5,089	2,115
670	60	7	8,856	2,510	6,025	4,690	1,208
			7,932	2,104	5,436	4,179	1,083
0	60*	1	947	304	610	0	577
0	110*	7	485	140	281	0	266

* Na I^{125} injected instead of I^{125}-BγG.

** Results obtained in duplicate rats given labelled BγG and single animals given Na I^{125}.

line in a gel-diffusion system (Fig. 4) suggested that a substantial part may have been intact antigen. No attempt was made to identify the label remaining in the pellet, but it is possible that this included additional antigenically active material. The relative amount of coprecipitable labelled material changed little; from 72 to 80% of the total extracted material, between 1 and 7 days. In animals injected with NaI^{125}, about two thirds of the label present in the thymus, 1 and 7 days later, was extracted by sonication but this was not precipitable in the specific immune system.

DISCUSSION

The present study shows clearly that soluble BγG penetrates the adult rat thymus and lymph nodes from the blood stream in a concentration comparable to that attained in other parenchymal organs such as the heart. Aggregated BγG penetrates these organs to a much more limited degree, if at all. From both thymus and node, protein disappears at a rate comparable to its rate of disappearance from other organs and the blood.[16,17] Over a substantial period of time, while a small amount appears actually to be within lymphoid cells, much more remain extracellular. Some of the soluble antigen and much of the aggregated is found within phagocytic cells, especially in the connective tissue stroma (capsule, vessel walls), and can be recognized autoradiographically in these areas and in phagocytic cells near vessels. In the thymus, more antigen is present in the medulla than the cortex. In newborn rats, there appears to be greater penetration, especially of aggregated materials, throughout the thymus, as well as the other organs studied.

These findings agree in detail with those of Mitchell and Nossal,[12,13] who recently studied the distribution of flagellin, polymerized flagellin, flagella, and BSA in rats of various ages, with regard to both the relative penetration into spleen, lymph node, and thymus in newborn and adult animals, the effect of state of aggregation of the test material, and the actual localization of antigen persisting within the thymus and nodes. It should now no longer be necessary to discuss the blood-thymus barrier as an absolute block to entry of antigen into the thymus from the blood stream and its hypothetical role in preventing immune responses within the thymus.[18] The penetration of proteins and a variety of other colloids into the thymus parenchyma, even in adult animals, is well established.[10,11,19,20] There is a barrier, nonetheless, which comprises the vascular endothelium and its basement membrane, a perivascular space containing collagen fibrils and pericytes, and the epithelial reticular cells and their basement membrane.[10,20] It is less in neonates[11,12,19] and in irradiated animals[21] than in intact adults.

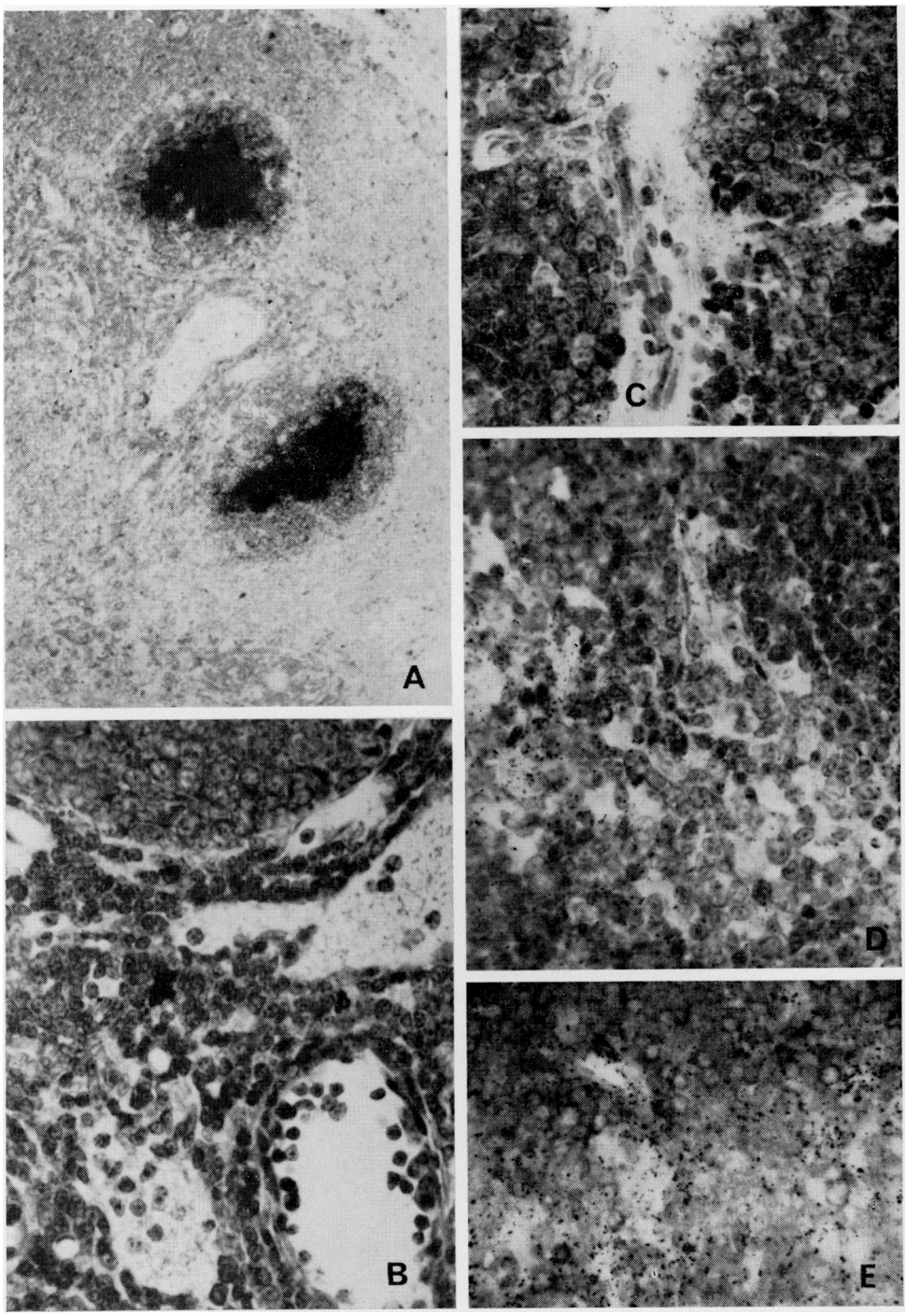

FIG. 3. Autoradiographs showing distribution of labelled BγG in lymphoid organs. (a.) Adult: Aggregated BγG accumulation in germinal centers of spleen 24 hours after intravenous injection. (b.) Adult: Pooling of soluble BγG in sinuses of lymph node 36 hours after intraperitoneal injection. Germinal center above. (c. and d.) Newborn: Accumulation of label in thymus parenchyma 7 days after intraperitoneal injection of crude BγG; c. shows increased concentration near cortical vessels; d. shows difference in relative concentration in cortex above and medulla below. (e.) Adult: Similar accumulation of antigen in thymus 24 hours after intraperitoneal injection of crude BγG. Again more is present in medulla (below) than in cortex.

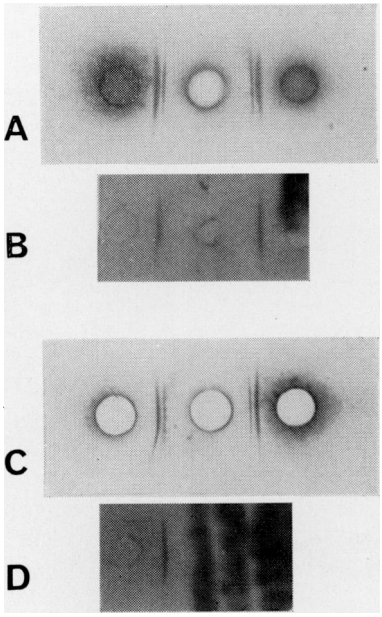

FIG. 4. Radioimmunodiffusion patterns obtained with adult thymus extracts one day (a., b.) and 7 days (c., d.) after intravenous injection of labelled crude BγG. The center well in each case contained the extract and the outer wells hyperimmune rabbit anti-BγG. a. and c. show the presence of two precipitin lines representing BγG and a β-globulin contaminant respectively. The autoradiographs (b. and d.) show the presence of label in the lines which correspond to BγG.

Its effect has been shown to be relative in all instances, affecting aggregated materials more than soluble and older animals more than younger.

The significance of the present findings lies in the probable role of antigen which enters the thymus in producing specific immunologic tolerance by direct interaction with thymocytes.[12,13] The thymus is recognized as the source of a population of lymphoid cells which play a role in immune responses of the delayed (cellular) type and formation of one or more types of humoral antibody in rats,[23-25] as well as in other species. They apparently play little or no role in formation of γM immunoglobulins, however.[23-25] These cells apparently come to the thymus from the bone marrow,[6,26] and later go on to seed peripheral lymphoid organs.[7] While in the thymus, they lack immunologic competence.[27,28] Our earlier studies have established that antigen entering the thymus, whether as soluble or aggregated protein or indeed in the form of living allogeneic cells, produces specific tolerance in the cells maturing there.[1-3,5] In animals lacking a peripheral pool of competent lymphocytes, shortly after birth or after irradiation, this may indeed be the major mechanism of tolerance induction.

In animals that possess a peripheral pool, interaction of antigen with peripheral lymphocytes must also occur for tolerance to be achieved. Such an interaction may take place in the blood or in peripheral organs such as lymph nodes. Extracellular antigen was found in the present study in the lymph node cortex and particularly pooled in sinuses within the cortex in adult rats. Both here and in the thymus, it appeared to be present as intact, macromolecular protein (see refs. 12,13). That it can interact with peripheral lymphocytes is shown by the fact that tolerance is produced by sufficient doses of antigen in normal adult animals,[4,29-33] even in the absence of the thymus.[8,9] When protein antigen is given by an immunizing route, there is evidence that it is taken up by phagocytic cells in which it becomes associated with intracellular organelles such as the lysosomes[34,35] or may form highly immunogenic complexes with a special form of RNA.[36-38] The induction of tolerance in adults appears to depend on bypassing of this phagocytic cell mechanism, as by the use of subimmunogenic doses of antigen,[39] antigen in a physical form which is not readily phagocytized,[29,30,40,41] or use of animals subjected to some form of blockade of their phagocytic cells.[42] Conversely, immunization is increased and the development of tolerance diminished where phagocytic uptake of antigen is enhanced. This occurs in animals with heightened reticuloendothelial function following treatment with mycobacterial adjuvant, endotoxin, or zymosan[4,30,43] or when artifically aggregated protein antigens are used,[4,29,30,40,41] proteins of large molecular size such as hemocyanin[44] or polymerized flagellin,[33,45] or indeed whole organisms[46] or parts of organisms,[33,45] or particulate adjuvant materials.[31,32,44]

It must be added, however, that aggregated materials occasionally produce some degree of tolerance as well.[4,31,32,47]

These considerations account for the relative ease with which tolerance, for any sort of antigen, may be induced in the neonatal period. At this time there is little blood-thymus barrier[11,12,19] (present study), the peripheral pool of lymphocytes is small or in some instances entirely lacking,[48] the antigen-trapping mechanism in peripheral lymphoid organs is deficient[12,13,49,50] and the antigen-trapping cells may actually lack certain necessary enzymes.[51,52] Thus, antigen fails to induce an immune response, persists for prolonged periods in the circulation, and has ready access to susceptible cells within the thymus. An explanation is also provided by these considerations for the difficulty that has accompanied attempts to induce transplantation tolerance in older animals, either with whole living cells[53] or disrupted cells or homogenates,[54] since these will in most instances be rapidly cleared by reticuloendothelial cells[17,55,56,57] and give rise to an immune response.

Tolerance, induced either in newborn animals or in adults, has been shown to reside in the peripheral pool of immunocompetent small lymphocytes.[4,9,58,59] We have considered above the conditions which favor direct interaction of antigen with these cells or with their precursors within organs like the thymus. Wherever it occurs, this reaction may take at most a few hours[9] with high doses of antigen; with minimal doses, it may take up to five days.[29,60] When labelled antigen is administered, it is subsequently found within lymphocytes, as well as in many other places.[61] Both Clark[10] and we have shown its presence within thymus lymphocytes. This localization of antigen however may have no relationship to the problem of tolerance induction, since quite large doses of material were injected in each instance. The Nossal group have stressed the need to make observations with smaller (physiologic) amounts of antigen, which may not be found in lymphocytes at all. Antigen remaining extracellular may be the principal agent producing tolerance. There is no evidence whether specific clones of cells are blocked, eliminated, or triggered to differentiation along an aberrant pathway in this process, or whether another type of effect, perhaps non-clonal in character, is involved.

SUMMARY

Crude bovine γ-globulin (BγG) and its heat-aggregated and "soluble" fractions, labelled with I^{125}, were injected in newborn, immature, and adult rats. The soluble preparation diffused readily into lymphoid and parenchymatous organs of both newborns and adults, and declined in concentration over a one-month period in parallel with the blood level of BγG. Aggregated BγG was rapidly taken up in reticuloendothelial organs (liver,

spleen, lung) and minimal amounts entered the adult thymus, lymph nodes, or heart. In the newborn, however, the amount of BγG entering these organs approached values obtained with soluble BγG. Fractionation and autoradiographic studies showed that most of the soluble antigen remained extracellular for long periods in both thymus and lymph node, particularly in the thymus medulla and in areas near blood vessels in both organs. Aggregated material tended to remain extracellular in newborn thymus and node, but in older animals, a substantial part was promptly taken up by phagocytic cells in the walls of vessels and the adjacent parenchyma. Values obtained in 4-5 week old rats were intermediate between those in newborns and adults, and values with crude BγG intermediate between those with soluble and aggregated material. At least half of the labelled material in the thymus 1 and 7 days after injection had the antigenic activity of BγG.

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