

Kinetics of cells producing antibodies to penicillin studied by plaque assay

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The Jerne plaque assay technique was used for the detection and examination of the kinetics of antibody production to penicillin in mice. Potassium penicillin G (KPG) was linked to hemocyanin (KLH). Swiss mice were given KPG-KLH intravenously. Spleens were removed one to ten days after immunization. Sheep red blood cells (S.R.B.C.) were sensitized with KPG. The spleen cells and KPG-S.R.B.C. were mixed and plated as in Jerne's method. After incubation and overlaying with guinea pig complement, plaques of immune hemolysis were counted. Controls consisted of spleen cells from nonimmunized mice or from animals given KLH only. Peak numbers of plaques with KPG-S.R.B.C. occurred on the fourth day after immunization. There was a thirty- to fortyfold increase in numbers of plaque-forming cells (PFC) in KPG-KLH immunized animals compared with background PFC in nonimmunized mice. A four- to eightfold increase over background was observed in PFC in KLH immunized mice. With this method, it appears possible to study the kinetics of antibody formation to a haptenic allergen such as penicillin.

The localized hemolysis-in-gel plaque-forming-cell (PFC) method¹ for detecting cells producing antibody to sheep red blood cells (S.R.B.C.) has been broadened by coupling antigens to the indicator red blood cells (R.B.C.). In addition to those native to R.B.C., such antigenic determinants as bacterial polysaccharides² and proteins,³ and such simple haptenes as arsanilic acid, sulfanilic acid, and dinitrophenol⁴ have been used for this purpose.

In order to develop a model for the investigation of antibody-producing cells

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Table 1. *Antibody production by single spleen cells from mice immunized with a volume of 0.2 ml.*

<i>Experiment</i>	<i>Plaque-forming cells/</i>			
	<i>First day</i>	<i>Second day</i>	<i>Third day</i>	<i>Fourth day</i>
<i>KPG-KLH</i>				
1	3	99	1,403	620
2	385	461	1,471	1,745
3	7		2,095	891
4			905	3,911
<i>Average</i>	132	280	1,469	1,792
<i>KLH</i>				
<i>Average</i>	3	115	375	190

*Number of plaque-forming cells from nonimmunized control mice subtracted.

from allergic individuals, we have studied the early immune response to penicillin in a laboratory animal. The PFC assay, with the use of penicillin coupled to S.R.B.C., was therefore used in mice for the detection and examination of the kinetics of antibody production to penicillin.

PROCEDURE

Materials used included crystalline potassium benzylpenicillin G* (KPG), Giant Keyhole Limpet Hemocyanin† (KLH), tissue culture medium 199‡ (TC 199), Difco Bacto Agar,§ DEAE Dextran,|| trimethylamine hydrochloride,¶ and rabbit anti-S.R.B.C. stroma.# Other chemicals were of reagent grade.

Potassium benzylpenicillin G (KPG), 12,000 U. or 1,200 U., was linked to 2 mg. KLH in 2 ml. 1 per cent saline by the method of Nakazima⁵ with incubation at 37° C. for 24 hours. The pH, which had dropped from 6 to 4.5 during incubation, was adjusted after alum precipitation to pH 8 with 0.1N NaOH.

Random-bred white Swiss mice, weighing 18 to 23 grams, were immunized with the KPG-KLH conjugate in 0.2 ml. volume intravenously (Table 1). An average experiment consisted of 2 mice injected with 1,200 U. KPG-KLH antigen (KPG-KLH concentrate), 2 injected with 120 U. KPG-KLH antigen (KPG-KLH dilution), and 2 injected with 0.2 mg. KLH in 1 per cent saline without KPG. One mouse which had not been injected was used for "background" control.

The PFC assay was modified from the method of Jerne and co-workers.⁶ The bottom layer consisted of 12 ml. of 1.5 per cent Difco Bacto Agar dissolved in TC 199. The cell layer was 2.5 ml. of 0.75 per cent Difco Bacto Agar in TC

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potassium penicillin G-Keyhole Limpet Hemocyanin conjugate (1,200 U.: 0.2 mg.) in

<i>1 × 10⁸ spleen cells*</i>					
<i>Fifth day</i>	<i>Sixth day</i>	<i>Seventh day</i>	<i>Eighth day</i>	<i>Ninth day</i>	<i>Tenth day</i>
242	212	91	59	4	64
224	87	524	825	178	13
1,812	9	669		0	
97	357				
594	169	428	442	67	39
173	35	69	109	87	3

199. Fresh guinea pig serum, absorbed with S.R.B.C. and diluted 1:10, was used as a source of complement for developing the plaques.

The mice were exsanguinated one to ten days after immunization, and the spleens were removed, washed in saline, transferred to TC 199, maintained at 4° C. in an ice bath, and then disrupted by gentle teasing with forceps. The spleen cells from identically treated mice were usually combined.* The cells in TC 199 were pipetted through a stainless steel mesh to remove clumps, washed once in TC 199, and kept at 4° C. until ready for plating.

Four to 14 day S.R.B.C. were sensitized with KPG by the method of Levine,⁷ and a new batch was made up each day.

The spleen cells and the KPG-S.R.B.C.† were mixed at 45° C. and plated according to the technique of Jerne and co-workers.⁶ Spleen cells per plate numbered 1×10^8 and 1×10^7 . After incubation at 37° C. for 1 hour followed by overlaying with fresh guinea pig serum diluted 1:10 and then another incubation for 30 minutes, plaques of immune hemolysis which had formed were counted‡ with a Bausch and Lomb Stereo-Zoom scope, BVB-73. Controls consisted of spleen cells from nonimmunized mice and cells from animals given 0.2 mg. KLH.

An antiserum against mouse 7S γ G-globulin (γ G) was induced in a rabbit which was immunized with γ G in Freund's adjuvant subcutaneously, followed by intravenous boosters of the alum-precipitated antigen. The mouse 7S γ G-globulin was prepared from pooled normal mouse sera by separation on a DEAE-cellulose column at pH 8, 0.01M phosphate buffer.⁸ This antiserum showed good precipitin bands with mouse γ G. After the plaques had been counted initially, the rabbit antimouse serum, diluted 1:50, was layered over the spleen cell KPG-S.R.B.C. layer with incubation, followed by reincubation with complement, in an effort to detect 7S plaques by the "development" technique of Dresser and Wortis.⁹

*In several experiments, numbers of PFC's from individual mice were determined.

†In addition to KPG-S.R.B.C. in several experiments nonsensitized S.R.B.C. were used for controls.

‡Courtesy of N. L. Petrakis, M.D., Dept. Epidemiology and International Health, University of California San Francisco Medical Center.

Passive cutaneous anaphylaxis (PCA) experiments were performed in Hartley strain male guinea pigs weighing 250 grams. by the method of Ovary.¹⁰ Sheep hemoglobin was prepared by lysis of S.R.B.C. with distilled water and washed five times by centrifugation. Hemoglobin-free S.R.B.C. stroma was prepared by the method of Komninos and Rosenthal.¹¹ Antihemocyanin precipitating antibody* was induced in rabbits immunized with 2 to 3 mg. KLH in Freund's adjuvant subcutaneously with intravenous booster doses.

The anti-KLH was injected into the skin of the guinea pig intradermally in dilutions of 1:10 and 1:100. Three hours later 0.25 ml. 1 per cent Evans blue dye was injected intravenously. After noting any nonspecific bluing, 90 mg. sheep hemoglobin was injected intravenously. The skin was observed for bluing for 2 hours. Then hemoglobin-free S.R.B.C. stroma, 0.15 ml. of precipitate from 10 ml. S.R.B.C. suspended in 1 ml. saline, was injected intravenously and the animals were observed for 2 more hours.

Rabbit anti-S.R.B.C. stroma was injected intradermally in 1:10 and 1:100 dilutions followed by intravenous Evans blue dye and 1 mg. hemocyanin 3 hours later.

RESULTS

Plaques were seen in increased numbers compared to "background" plaques from nonimmunized control mice on plates with spleen cells from mice immunized with KPG-KLH and challenged with KPG-S.R.B.C. It appeared that these plaques were specific for KPG since the numbers observed from spleen cells immunized against KPG were many times greater than those from nonimmunized mice. By light microscopy, the center of each plaque had a single lymphoid cell surrounded by a clear zone of S.R.B.C. lysis.

Peak numbers of plaques with KPG-S.R.B.C. occurred on the third and fourth days after immunization. Fig. 1 shows the responses from the first day (24 hours) to the tenth day after injection. The results of PFC's from mice immunized with KPG-KLH concentrate and KPG-KLH dilution were similar, and the numbers of plaques were comparable. Some variation occurred from the sixth to the ninth days. Nonimmunized control mice had "background" PFC's from 3 to $89/1 \times 10^8$ spleen cells (average: 45) when challenged with KPG-S.R.B.C. These daily values were subtracted from the test-plate total numbers of PFC's. When spleen cells from mice immunized with KPG-KLH and KLH were challenged with nonsensitized S.R.B.C., PFC responses were in the same range as "background" seen in nonimmunized mice challenged with KPG-S.R.B.C. or S.R.B.C.

Another group of control mice was immunized with KLH only and was challenged with KPG-S.R.B.C. Surprisingly, the results were not unlike those of the KPG-KLH mice but had approximately 1/6 the number of specific KPG PFC's. The KPG-KLH concentrate mice showed a peak response on the third and fourth days 32 to 40 times greater, the KPG-KLH dilution mice 25 to 34 times greater, than the nonimmunized control mice; while the control mice

*Several antisera were supplied through the courtesy of Dr. Frank J. Dixon, Scripps Clinic and Research Foundation, La Jolla, Calif.

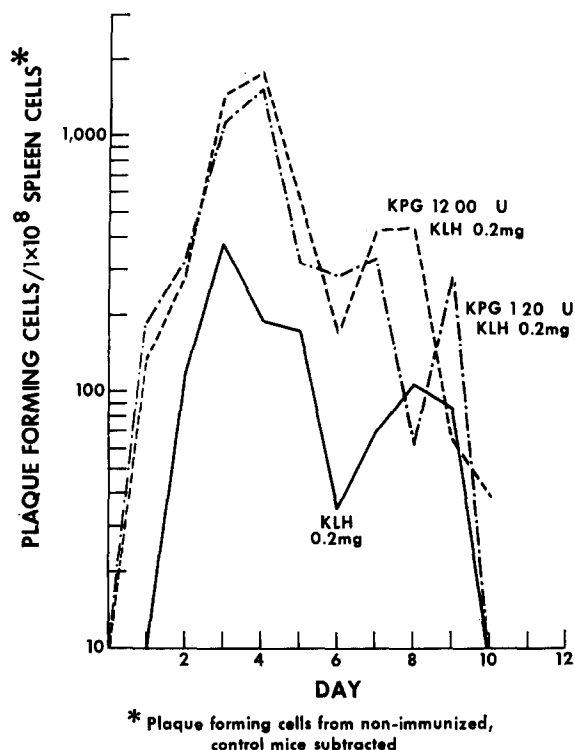


Fig. 1

Antibody production by single spleen cells immunized with potassium penicillin G-Keyhole Limpet Hemocyanin (KPG-KLH) or Keyhole Limpet Hemocyanin (KLH).

immunized with KLH alone peaked at values of 4 to 8 times those of nonimmunized controls. Considerable variation, generally within one-log range, was observed from different mice on the same days following immunization.

The cellular responses in animals immunized with KPG-KLH and KLH alone were somewhat biphasic with major peaks on the third and fourth days and a second smaller rise on the seventh and eighth days.

Tables I through III show numbers of PFC's on individual days after immunization with the results for each experiment and the mean values on Fig. 1. By the sixth day there was a definite decrease in PFC's for KPG-KLH concentrate and dilution which, after a second but lower rise, continued to the tenth day. A similar pattern was observed for KLH PFC's.

The results of experiments with rabbit antimouse γ G to develop γ G PFC's on the eighth, ninth, and tenth days revealed no increase in PFC's over those counted after the initial challenge and incubation with guinea pig serum.

In view of the increased numbers of PFC's over background from mice immunized with KLH and later challenged with KPG-S.R.B.C. PCA experiments were performed to try to demonstrate cross-reactivity between the induced hemocyanin antibody(ies) and antigen (or antigens) inherent to the S.R.B.C. membrane or to sheep hemoglobin. Table IV shows the results of PCA experi-

Table II. *Antibody production by single spleen cells from mice immunized with of 0.2 ml.*

<i>Experiment</i>	<i>Plaque-forming cells/</i>			
	<i>First day</i>	<i>Second day</i>	<i>Third day</i>	<i>Fourth day</i>
<i>KPG-KLH</i>				
1	58	166	1,885	1,463
2	52	457	1,025	1,127
3	435		1,490	1,951
4			260	
<i>Average</i>	182	312	1,125	1,514
<i>KLH</i>				
<i>Average</i>	3	115	375	190

*Number of plaque-forming cells from nonimmunized control mice subtracted.

Table III. *"Cross-reacting" or "background" antibody production by single spleen cells*

<i>KLH control experiment</i>	<i>Plaque-forming cells/</i>			
	<i>First day</i>	<i>Second day</i>	<i>Third day</i>	<i>Fourth day</i>
1	4	76	538	109
2	2	154	443	140
3			406	487
4			111	22
<i>Average</i>	3	115	375	190

*Number of plaque-forming cells from nonimmunized control mice subtracted.

Table IV. *Passive cutaneous anaphylaxis in the guinea pig*

<i>Antisera</i>	<i>Dilution</i>	<i>Sheep Hgb.</i>			<i>S.R.B.C. stroma</i>			<i>KLII</i>	
		<i>Animal 1</i>	<i>Animal 2</i>	<i>Animal 3</i>	<i>Animal 4</i>	<i>Animal 5</i>	<i>Animal 6</i>	<i>Animal 7</i>	<i>Animal 8</i>
<i>Anti-KLH</i>									
1	1:10	0			4+				
	1:100	0			3+				
2	1:10	0			2+				
	1:100	0			2+				
3	1:10	0			4+				
	1:100	0			4+				
4	1:10		0			3+			
	1:100		0			0			
5	1:10		0			2+			
	1:100		0			0			
6	1:10		0	0		2+	1+		
	1:100		0	0		1+	1+		
7	1:10			0			4+		
	1:100			0			4+		
8	1:10			0			4+		
	1:100			0			3+		
<i>Anti-S.R.B.C. stroma</i>									
9	1:10							2+	3+
	1:100							1+	1+
10	1:10							2+	2+
	1:100							2+	1+

The abbreviations are: 0 = 5 mm.; 1+ = 5 to 9 mm.; 2+ = 10 to 14 mm.; 3+ = 15 to 19 mm.; and 4+ = 20 mm.

potassium penicillin G-Keyhole Limpet Hemocyanin (120 U.: 0.2 mg.) in a volume

<i>1 × 10⁸ spleen cells*</i>					
<i>Fifth day</i>	<i>Sixth day</i>	<i>Seventh day</i>	<i>Eighth day</i>	<i>Ninth day</i>	<i>Tenth day</i>
546	611	167	75	45	0
224	105	811	50	340	18
294	29	29		504	
233	380				
325	282	336	63	296	9
173	35	69	109	87	3

from mice immunized with Keyhole Limpet Hemocyanin 0.2 mg.

<i>1 × 10⁸ spleen cells*</i>					
<i>Fifth day</i>	<i>Sixth day</i>	<i>Seventh day</i>	<i>Eighth day</i>	<i>Ninth day</i>	<i>Tenth day</i>
188	70	115	38	23	0
45	35	87	181	52	6
458	0	6		186	
0	35				
173	35	69	109	87	3

ments performed with different rabbit antisera to hemocyanin and S.R.B.C. stroma.

Washed S.R.B.C. stroma induced PCA reactions in guinea pig skin sensitized with rabbit anti-KLH sera. Stroma-free sheep hemoglobin failed to induce such reactions. KLH-induced PCA reactions in guinea pig skin sensitized with rabbit anti-S.R.B.C. stroma.

DISCUSSION

It is possible to study the kinetics of antibody formation to a haptenic allergen such as penicillin by the localized hemolysis plaque (PFC) assay. By this technique, spleen cells immunized with KPG-KLH and challenged with KPG-S.R.B.C. reveal hemolysin production which is specific for penicillin from two to nine days after immunization.

The diastereoisomeric mixture of benzylpenicilloyl (BPO) groups has importance because these groups are antigenic determinants responsible for immediate hypersensitivity reactions to penicillin in man.¹² Sensitization of red blood cells at an alkaline pH for the detection of hemagglutinating antibody identifies predominately antibodies for this group of haptenes.⁷ The specific hapten (or haptenes) of KPG which interacts with KLH at an acid pH was not determined in the present study. Because the BPO group is formed with KPG-S.R.B.C. at an alkaline pH in hemagglutination studies, presumably the BPO group is also the responsible immunogen for the lytic reaction in these experiments. That KPG-specific PFC's can result from immunization with KPG

conjugated to various proteins, including KLH, at an alkaline pH has recently been reported in rabbits.¹³

Our previous attempts, with the use of egg albumin and bovine gamma globulin as carriers for KPG given intravenously, were unsuccessful for demonstrating PFC's. In initial experiments with KLH and a concentration of KPG of approximately 30,000 U. per mouse, fewer PFC's were noted on KPG-KLH plates than on KLH controls. Immunization with various dilutions of KPG conjugated to 2 mg. KLH revealed that KPG-KLH concentrate (1,200 U.) and KPG-KLH dilution (120 U.) gave optimal results. With the larger concentrations of penicillin, free KPG may have been present to tie up the "preformed" antibody necessary for induction of antibody synthesis by KPG-KLH.

KLH was used as carrier for KPG hapten (or haptenes) because of its large molecular size (7,500,000 mol. wt.). As prepared with a final alkaline pH, it was presumably in the dissociated form (814,000 mol. wt.).¹⁴

The detection of "weak-reacting" 7S hemolysin by the plaque technique has been demonstrated for both the S.R.B.C. antigen system^{9, 15} and haptenes conjugated with S.R.B.C.⁴ The biphasic nature of PFC response from animals immunized with both KPG-KLH and KLH alone suggested that the second peak on the seventh and eighth days might be due to 7S plaque-forming cells; however, biphasic responses were noted by Wortis and colleagues¹⁶ with both 19S and 7S PFC's after immunization with S.R.B.C. A possible explanation for a biphasic primary response might come from an analogy to the data of Jacobson and Thorbecke¹⁷ in which they observed, upon secondary antigenic stimulation, an initial antibody response that came from the splenic white pulp, followed several days later by an additional contribution from the splenic red pulp.

From the observations on the eighth to tenth days in the present study, with the use of the "development" technique with rabbit anti-7S mouse γ G-globulin, we were unable to detect "weak-reacting" hemolysin to KPG-KLH. However, the technique was limited by the use of only one dilution of antiserum, and the quantitative antibody content of this serum was not determined. The failure to demonstrate 7S PFC's would suggest that the antibody evoked by KPG-KLH was predominately 19S. This is supported by the observations of Dixon and colleagues¹⁸ that the antibody responses of rabbits and rats to KLH differ from their response to other proteins in that a considerable amount of the resulting antibody in both the primary and secondary responses is mercaptoethanol-sensitive. An alternative for the failure to demonstrate 7S PFC's might be due to the relatively low hemolytic efficiency of 7S as compared with 19S antibody.¹⁹

Others using the hemolytic plaque assay^{1-3, 16} have found considerable variation in PFC response by individual animals, varying generally within a one-log range. A possible explanation for this in the present study is that the mice were random-bred white Swiss with a wide individual variety of immunological capacity. Inbred mice might show less variation to a given antigen.²⁰

The much smaller (10 to 60 times less) PFC response noted to KPG-KLH as compared to the S.R.B.C. antigen system,^{6, 21} indicates the relative weakness of the antigenic stimulus from this hapten-carrier complex. This finding has been confirmed with different haptenes.²²

The demonstrations by PCA of cross-reactivity between KLH and S.R.B.C.

stroma is being studied further. One may speculate as to common antigens on the two proteins which may have some common functional significance.

As a model for the study of hypersensitivity reactions and the origin of antibodies responsible for such reactions in man, the localized hemolysis plaque technique in animals has been broadened to include penicillin. With this demonstration of another means of studying the immune mechanisms of this clinically important haptene, it is hoped that this technique will be used for the study of penicillin allergy in man.

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