STUDIES ON THE Cx-REACTIVE PROTEIN

III. THE EFFECT OF IRRADIATION OF RABBITS ON THE ACUTE PHASE PROTEIN SYSTEM* †

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The occurrence in the rabbit of an abnormal protein analogous in many of its properties to the human C-reactive protein has been demonstrated (1). This rabbit acute phase protein reacts only with a special form of the pneumococcal somatic polysaccharide, Cx, whereas human C-reactive protein reacts both with Cx and the classical C polysaccharide. Both the human and the rabbit proteins as they occur in the blood are bound to lipid and in both instances the lipid-protein complexes are precipitable by calcium ion if the salt concentration is low.

Both acute phase proteins are antigenic in several laboratory animals (1–3). Lipid-free preparations of them, crystalline or not, act as antigens which stimulate in sheep the production of specific antisera which do not react with normal serum. Reciprocal immunological cross-reactions employing sheep antisera to C- and Cx-reactive proteins have recently been demonstrated (3). Although these are partial cross-reactions, they are appreciable.

Virtually nothing is yet known concerning any natural biological function of these acute phase proteins to explain their appearance and disappearance from the blood. There is, however, some evidence that the Cx-reactive protein is elaborated in response to appropriate stimuli by some cellular component of the reticulo-endothelial system (4). Blockade of the reticulo-endothelial system with thorotrast reduces the capacity of rabbits to elaborate the abnormal protein.

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Experiments have been carried out in an attempt to elicit the Cx-reactive protein response in rabbits by the use of agents which do not produce an inflammatory reaction, but which have a direct cytotoxic effect on the bone marrow and the lymphoid tissues (5, 6). It was found that x-radiation and one of the radiomimetic compounds caused the appearance of Cx-reactive protein 24 hours after exposure.

The present investigation was undertaken to determine the pattern of Cx-protein response caused by varying doses of radiation to rabbits and its possible relation to their susceptibility to subsequent endogenous bacteremia. The effect of total body exposure to X or gamma radiation on the susceptibility of rabbits and mice to bacterial infection has been the subject of inquiry by the junior authors for a number of years (7–9). Postirradiation infection of enteric origin was found to occur in rabbits following irradiation with 900 r, but serial blood cultures during life and culture of organs at autopsy were often required to demonstrate such infection.

**EXPERIMENTAL PROCEDURE**

Three general types of experiments were carried out. In the first, rabbits were exposed to a single dose of x-radiation which ranged from 50 to 1200 r. Daily serial bleedings were tested for the presence of Cx-reactive protein. In the second set of experiments autopsy cultures were made on rabbits that died or were sacrificed at different times post irradiation. In the third set of experiments, irradiated rabbits in which an initial Cx-protein response had subsided, were inoculated intravenously with live or killed suspensions of Escherichia coli.

**Materials and Methods**

*Preparation of Sheep Antiserum to Cx-reactive Protein.*—Precipitating antiserum of high titer was obtained from a sheep 4 weeks following the intracutaneous injection into 6 separate skin sites of purified Cx-reactive protein incorporated into complete adjuvant. The immune serum which gave a slight cross-reaction with normal rabbit serum was absorbed with dried normal rabbit serum.

*Precipitin Tests with the Antiserum.*—The precipitin tests with antiserum to Cx-reactive protein were carried out in capillary tubes according to the procedure described by Anderson and McCarty (10). This method is based on the capillary precipitin method for serological typing of Group A streptococci described by Swift, Wilson, and Lancefield (11). The quantity of precipitate formed was measured in millimeters.

*Rabbits.*—The rabbits were healthy young adults, weighing 2 to 3 kg, obtained from a snuffle-free colony. After arrival at the laboratory they were housed in separate cages in a room in which other animals were excluded, and were cared for by a man who had no contact with other rabbits. Food and water were available at all times.

*Bleedings.*—Most of the rabbits were bled every day (except Sundays) beginning the day before irradiation. Five which were found to have positive reactions before irradiation were

1 Maintained at Lakeside, Michigan, by Mr. Harold H. Swift for the benefit of investigators at the University of Chicago.
discarded. Blood was drawn aseptically from a marginal ear vein into a small syringe, allowed to clot overnight in a centrifuge tube, and centrifuged the following day. The serum was removed and stored in the refrigerator.

Irradiation.—The animals were exposed to x-rays from a General Electric maxitron 250 at the Argonne Cancer Research Hospital. They were placed in perforated aluminum boxes of proper size to prevent their moving about. The radiation dose was delivered half to one side of the body and half to the other. The x-ray factors were: 250 kv., 30 ma., 0.5 mm. Cu and 1 mm. Al filter; approximately 45 r per minute; distance from tube to target (midpoint of rabbit) 79 to 80 cm.

Autopsy Cultures.—The chest cavity was opened aseptically and 1 or 2 ml. of blood aspirated from the heart. A drop was cultured on plates of blood agar and eosin-methylene blue agar, the remainder in brain-heart infusion broth. The abdominal cavity was then opened aseptically and 6 pieces of the liver (totaling about /3 of the whole) were excised from different parts. Areas showing any gross pathological change were always included. The pieces of liver were cultured in 50 ml. of brain-heart infusion broth. The spleen was removed, cut in several places, and dropped into a flask of broth. The lungs of most of the sacrificed rabbits were cultured by the same procedure as the liver. After 24 and 72 hours incubation, subcultures were made onto appropriate diagnostic media.

Bacterial Suspensions Used for Intravenous Inoculation.—A streptomycin-resistant strain of E. coli, of rabbit origin, was grown for 24 hours on nutrient agar, suspended in saline, and diluted to contain approximately \(10^8\) bacilli per ml. The numbers of viable bacteria were confirmed by plating, in quadruplicate, 0.1 ml. of the \(10^{-6}\) and \(10^{-7}\) dilutions.

Suspensions of killed bacteria were prepared from the same strain of E. coli in the same manner, killed by heating in boiling water for 30 minutes, washed three times by centrifugation, and, using a Coleman spectrophotometer, diluted to contain approximately \(10^9\) bacilli per ml.

RESULTS

The appearance of Cx-reactive protein in the blood of irradiated rabbits was usually a diphasic phenomenon. It first appeared 24 to 48 hours after irradiation, persisted 2 to 4 days and then disappeared. This initial appearance, designated the primary phase, was observed in all but 5 of 97 rabbits exposed to 500 r or more, but in only 4 of 16 rabbits exposed to 200 r or less (see Table I). Ten of 14 rabbits irradiated with 1200 r died during the primary phase as did 9 of 43 irradiated with 900 r.

The primary phase which lasted 2 to 4 days was followed by a negative phase; i.e., a period in which Cx-reactive protein was absent from the blood. It reappeared during the 2nd week post irradiation in many, but not all the rabbits exposed to the two highest doses of x-ray (900 and 1200 r). This secondary phase occurred in only 2 of 13 rabbits in the 700 r series and in 2 of 20 exposed to 500 r or less.

Fig. 1 shows illustrative examples of the various patterns of response which
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followed exposure to 900 r. The amount of Cx-reactive protein in each serum is recorded as millimeters of precipitate formed.

Rabbits A and B showed only the primary phase, which varied in duration from 1 to 4 days. Rabbit A was sacrificed on the 9th day for autopsy cultures, all of which showed no growth. Rabbits C and D showed both primary and secondary phases with a negative phase intervening. Rabbit C which was sacrificed during the secondary phase was found to have a generalized infection with \textit{E. coli}. Rabbit D was sacrificed for autopsy cultures on the 22nd day after the secondary phase. Growth occurred in none of its cultures.

\textit{Effect of Second Irradiation with 900 r.}—Three rabbits which had been exposed to 900 r and allowed to recover were irradiated a 2nd time with 900 r on the 49th day. Cx-reactive protein promptly reappeared in their blood, as it had following the initial exposure.

\textbf{TABLE I}

\textit{Appearance of Cx-Reactive Protein Following Various Doses of Irradiation}

<table>
<thead>
<tr>
<th>Irradiation dose</th>
<th>No. of rabbits tested</th>
<th>No. rabbits Cx positive in:</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Primary phase</td>
<td>Secondary phase</td>
</tr>
<tr>
<td>1200 r</td>
<td>14</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>900 r</td>
<td>43</td>
<td>42</td>
<td>28</td>
</tr>
<tr>
<td>700 r</td>
<td>36</td>
<td>32</td>
<td>2 of 13*</td>
</tr>
<tr>
<td>500 r</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>200 r</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>100 r</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>50 r</td>
<td>8</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

* All but 13 of the 700 r rabbits were used in the intravenous inoculation experiments.

\textit{Results of Autopsy Cultures}.—In the 900 r series no growth occurred in cultures of heart's blood, liver, spleen, or lung from 6 rabbits sacrificed during the primary phase and 1 which died during the intermediate or negative phase. Nor were any positive cultures obtained from six rabbits sacrificed for this purpose and one which died during the post-secondary period; i.e., after the 2nd disappearance of Cx-reactive protein.

Bacteria were recovered, however, from all of the 15 animals which died and 6 of 9 sacrificed during the secondary phase. In other words, bacterial infection was demonstrated in all but 3 of 24 rabbits autopsied at this time. It is possible that more exhaustive cultures of organs might have revealed a focus of infection in these three rabbits. Earlier studies showed that the bacteria present in organs of irradiated (900 r) rabbits might be few in number (7). The results of the autopsy cultures are summarized in Table II.

The microorganism most frequently recovered in autopsy cultures was \textit{E. coli} which was found in 14 rabbits. An unidentified Gram-negative bacillus
was recovered from 3. Less frequently found, sometimes associated with *E. coli*, were *Staphylococcus aureus*, a spore-forming rod, and an alpha *Streptococcus*. When bacterial infection was present, it was always found in the liver, and usually in the spleen and blood. With very few exceptions, the same microorganism or pair of microorganisms was recovered from all cultures of an infected rabbit.

These results showed that the secondary, but not the initial appearance of Cx-reactive protein in the blood of irradiated rabbits was usually associated with bacterial infection.

**Attempt to Suppress the Secondary Phase by Treatment with Antibiotics.**—50 mg. of penicillin and 100 mg. of dihydroxystreptomycin\(^3\) administered intramuscularly twice daily failed to prevent the reappearance of Cx-reactive protein in the blood of 4 rabbits illustrating different patterns of response to irradiation with 900 r.

\(^3\) The antibiotics used in these experiments were generously supplied by the following: Lederle Laboratories, Pearl River, New York. Charles Pfizer and Company, Inc., Brooklyn, New York. E. R. Squibb and Sons, New Brunswick, New Jersey.
protein, presumably because the injections themselves produced sufficient trauma to stimulate its appearance. This explanation is based on the observation that similar injections into non-irradiated rabbits caused the appearance of Cx-reactive protein.

Response to Intravenous Inoculation of Viable Bacteria during the Negative Phase.—The following experiments were performed to answer two questions: (a) did Cx-reactive protein disappear after the primary phase because the animals were no longer able to produce it, i.e., was the negative phase due to inability to elaborate Cx-reactive protein? and (b) was the reappearance (secondary phase) caused by endogenous bacterial infection?

TABLE II
Results of Autopsy Cultures and Tests for Cx-Reactive Protein at Different Times Post Irradiation (900 r)

<table>
<thead>
<tr>
<th>Phase</th>
<th>Time post irradiation</th>
<th>Rabbits autopsied</th>
<th>Cx reaction at death or sacrifice</th>
<th>Culture results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>1st–5th</td>
<td>6 sacrificed</td>
<td>6 positive</td>
<td>6 negative</td>
</tr>
<tr>
<td>Negative</td>
<td>3rd–11th</td>
<td>1 died</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Secondary</td>
<td>4th–12th</td>
<td>15 died</td>
<td>15 positive</td>
<td>15 positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9 sacrificed</td>
<td>9 positive</td>
<td>6 positive, 3 negative</td>
</tr>
<tr>
<td>Post Secondary</td>
<td>13th–21st</td>
<td>1 died</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 sacrificed</td>
<td>6 negative</td>
<td>6 negative</td>
</tr>
</tbody>
</table>

Small groups of rabbits were irradiated with 900 or 700 r and tested daily for the presence of Cx-reactive protein. The primary phase was demonstrated in all of them. After a rabbit had become negative, i.e., lost Cx-reactive protein from its blood, it was inoculated intravenously with a suspension containing $10^8$ viable bacteria ($E. coli$). Cx-reactive protein reappeared in the blood of every rabbit within 24 hours,—in many of them as early as 6 hours after infection. Two rabbits which were inoculated after a long period of negative tests, on the 47th day post irradiation, responded in a similar fashion. Cx-reactive protein persisted in the blood of every rabbit until it died or was sacrificed to terminate the experiment.

All of the rabbits irradiated with 900 r and inoculated with $10^8$ $E. coli$ died, except one which was sacrificed and the two which were inoculated on the 47th day post irradiation; i.e. after they had recovered from radiation injury. Deaths were less numerous among the 700 r rabbits inoculated with viable bacteria. The responses of rabbits challenged with intravenous injection of viable or dead bacteria are summarized in Table III.
Response to Injection of Killed Bacteria.—Ten rabbits irradiated with 700 r were injected intravenously with $10^9$ killed E. coli on the 3rd or 4th day post irradiation. Cx-reactive protein appeared in the blood of each 24 hours after inoculation (none was tested at 6 hours) but disappeared again after 24 to 48 hours. In three of these rabbits, it reappeared 7 days after inoculation and disappeared 2 days later.

TABLE III
Appearance of Cx-Reactive Protein Following Intravenous Injection of Viable or Dead Bacteria (E. coli) during Negative Phase

<table>
<thead>
<tr>
<th>Radiation dose</th>
<th>Bacteria injected</th>
<th>Time of injection post irradiation</th>
<th>No. rabbits</th>
<th>Duration of positive reactions*</th>
<th>Day of death post irradiation</th>
<th>Days observed post irradiation</th>
<th>No. surviving to end of experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>900 r</td>
<td>$10^9$ viable</td>
<td>5th</td>
<td>8</td>
<td></td>
<td>6, 7, 8, 8, 9, 9, 9, 9</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9th</td>
<td>4</td>
<td>Until death or termination</td>
<td>10, 11, 24</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47th†</td>
<td>2</td>
<td></td>
<td>—</td>
<td>78</td>
<td>2</td>
</tr>
<tr>
<td>700 r</td>
<td>$10^9$ dead</td>
<td>3rd</td>
<td>10</td>
<td></td>
<td>6, 8, 11, 15</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4th</td>
<td>4</td>
<td></td>
<td>10, 17</td>
<td>19</td>
<td>2</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>—</td>
<td>8</td>
<td></td>
<td>9</td>
<td>16</td>
<td>7</td>
</tr>
<tr>
<td>700 r</td>
<td>$10^9$ dead</td>
<td>3rd</td>
<td>4</td>
<td>1 to 2 days</td>
<td>—</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4th</td>
<td>6</td>
<td>1 to 2 days</td>
<td>13</td>
<td>19</td>
<td>5</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>—</td>
<td>3</td>
<td>1 to 4 days</td>
<td>—</td>
<td>18</td>
<td>3</td>
</tr>
</tbody>
</table>

* All became positive 6 to 24 hours after injection.
† These 2 rabbits were in the period of recovery from radiation injury.

Three normal rabbits were injected intravenously with the same suspension of killed E. coli. The sera of all three contained Cx-reactive protein 24 hours after inoculation. In two rabbits it disappeared within 48 hours but not until the 5th day in the third. The secondary phase did not occur in any of the normal rabbits.

DISCUSSION

These findings demonstrate that the appearance of Cx-reactive protein in the blood of rabbits can be elicited by exposure to ionizing radiation as well as by bacterial infection. In rabbits irradiated with 900 r, the pattern of response was usually biphasic. The initial appearance of the protein within 48 hours of exposure was followed by a secondary appearance after an intermediate period of several days during which it was absent from the blood. This secondary response was associated with bacterial infection, either spontaneous or induced.

These observations give no indication of a possible biological function of
the acute phase protein. They emphasize, however, the importance of identifying the inciting agent or process responsible for its appearance, since many types of injuries have been shown to elicit it. Even in carefully controlled laboratory experiments, caution must be exercised in ascribing the appearance of Cx-reactive protein to a single known cause.

SUMMARY

Young adult rabbits (2 to 3 kg.) were subjected to a single total body exposure of x-radiation ranging from 50 to 1200 r and tested frequently for the presence of Cx-reactive protein in their blood.

It usually appeared in two phases separated by an interval of several days. The primary phase occurred 24 to 48 hours after irradiation in almost all (92 of 97) rabbits exposed to 500 r or more and in 4 of 16 exposed to 300 r or less. The secondary phase occurred during the 2nd week in many of the rabbits irradiated with 900 r or more and in a few irradiated with 700 r.

Autopsy cultures failed to demonstrate the presence of infection in rabbits which died or were sacrificed during the primary phase. Bacterial infection was demonstrated, however, in almost all (21 of 24) rabbits autopsied and cultured during the secondary phase.

After the disappearance of the primary phase in rabbits exposed to 700 or 900 r, the secondary phase could be elicited by initiating bacterial infection. Within 6 to 24 hours after intravenous inoculation of E. coli, Cx-reactive protein reappeared in the blood and persisted until death or termination of the experiment. Reappearance of the protein also followed the intravenous injection of killed E. coli but it disappeared again 1 to 2 days later.

The results indicate that the primary phase is elicited by radiation injury per se and the secondary phase by bacterial infection.

BIBLIOGRAPHY


