The Effect of Chromium on Platelet Function In Vitro

By HERMAN E. KATTLOVE AND THEODORE H. SPAET

Radioactive hexavalent chromium is a widely used agent for labeling platelets and studying their survival. Since platelet viability appears to be unaffected by chromium labeling, it is assumed that platelet function also remains intact. This assumption is basic to interpretation of platelet survival studies with chromium labeling. Accordingly, we examined the in vitro function of platelets that had been treated with sodium chromate. It was found that this agent inhibits several aspects of platelet function.

MATERIALS AND METHODS

Platelet-rich plasma (PRP) was prepared from normal volunteers who allegedly were taking no drugs. Blood, collected in 1/10 volume of either 3.8 per cent sodium citrate or one per cent EDTA in 0.85 per cent saline, was centrifuged for 15 minutes at 1600 RPM in a clinical centrifuge; the supernatant PRP was recentrifuged for 10 minutes at 800 RPM to remove contaminating red cells.

All containers and pipettes used for handling PRP or platelets were either plastic or polypropylene, except for the aggregometer cuvettes which were untreated glass.

The platelet aggregating agents were as follows:

a. Connective tissue fragments (CT) were prepared from human subcutaneous fat by a modification1 of the method described by Zucker and Borelli.2 For use as an aggregating agent, the final product was diluted in imidazole-buffered saline (IBS, pH 7.3) to the lowest concentration which caused brisk platelet aggregation. This is termed dilute CT. For other uses, the final wash product from approximately 50 Gm. of subcutaneous fat was suspended in 15 ml. of IBS. This turbid suspension is termed concentrated CT. Both products were stored in small aliquots in plastic tubes at -20°C, and were thawed just before use. No loss of activity was noted during the period covered by the experiments.

b. Adenosine diphosphate (ADP, Sigma) was dissolved in IBS and was stored at -20°C. After thawing, this was diluted to that concentration which would cause a secondary wave of aggregation3 or slow return to baseline (final concentration = 2-5 µM.).

c. Epinephrine (Parke-Davis) was diluted in IBS so that its final concentration when added to PRP was 4.5 × 10⁻⁷ M. Fresh epinephrine solution was prepared every half hour to avoid the deterioration that normally occurs.

d. Thrombin (commercial, Parke-Davis) was dissolved in IBS to a concentration which produced a standard thrombin time of about 20 seconds in citrated plasma.

Platelet aggregation was studied in a turbidometric4 system with continuous recording (Chrono-Log Aggregometer attached to Photovolt Densicord recorder). One-ml. samples of PRP were pipetted into a glass cuvette containing a teflon coated stir bar. The cuvette was placed in the aggregometer where the PRP was stirred at 1000 RPM and was kept at 37°C. One-tenth ml. of the aggregating agent was added, and the decrease in optical density of the PRP was recorded.

The effect of hexavalent chromium on platelet aggregation was tested by adding sodium chromate, dissolved in 0.85 per cent saline, to an aliquot of citrated PRP pooled from...
several ABO compatible donors. A second aliquot, to which an equal volume of saline was added, was used as a control. The pH of the PRP was unaffected by these additions. The aliquots were placed in a water bath at 37°C and a timer started. At measured intervals, alternate samples of chromated and control PRP were tested in the aggregometer by the addition of the aggregating agent. The maximum velocity of each aggregation curve was estimated by measuring the slope (ΔO.D./time in sec) of a straight line drawn through the steepest part of the curve. The slope of each chromium curve was compared with the slope of the succeeding control curve. Inhibition was calculated by the expression: 

\[ \text{per cent inhibition} = \frac{\text{control slope} - \text{chromium slope}}{\text{control slope}} \times 100 \]

The effect of chromate on platelet adhesion to concentrated connective tissue was studied in the aggregometer and monitored by continuous recording, as described by Spaet and Lejinieks.\(^5\) PRP was prepared from blood which was anticoagulated with EDTA to prevent platelet aggregation. The PRP was incubated for one hour at 37°C with either sodium chromate or saline. Samples were placed in the aggregometer cuvette, and 0.1 ml of the concentrated CT was added. As the platelets adhered to the CT fragments, the number of free platelets in suspension decreased, with a consequent fall in the O.D. of the PRP. The presence of platelet adhesion to CT and the absence of aggregation was confirmed by phase microscopy.

The CT-induced release of platelet adenine nucleotides was measured by utilizing their characteristic UV light absorption maximum of 258 mÅ.\(^6\) PRP, anticoagulated with EDTA, was incubated with sodium chromate in saline, or an equal volume of saline alone, for 90 minutes at 37°C. The PRP was divided into several 5-ml aliquots; the platelets were sedimented in a clinical centrifuge at 2500 RPM for 30 minutes, suspended in 0.1 per cent EDTA in Gaintner's buffer (pH 7.4) and resedimented. This “washing” was repeated twice and the platelets were suspended in 5 ml of the wash solution. The platelets in suspension were counted by phase microscopy. One-half ml of concentrated CT suspended in Sorenson’s buffer (pH = 8), or buffer alone was added, and the mixtures (final pH = 7.6) were tumbled for 10 minutes at room temperature on a vertical turntable. The specimens were centrifuged in a Clay Adams Sero-fuge for 10 minutes. The supernatants were harvested and cleared by recentrifugation at 25,000 RPM for one hour in a Spinco model L ultracentrifuge. The optical densities of the supernatants were measured at 258 mÅ in a Beckman DB spectrophotometer. The O.D. of the control (buffer alone) supernatants were subtracted from those of the CT supernatants. The final results were corrected for the initial platelet count of the suspension. Continuous scanning of the supernatants from 340 to 240 mÅ revealed no absorption peaks other than 258. The effect of chromate on platelet adenine nucleotide content was measured in a separate experiment. PRP was incubated for 90 minutes at 37°C with either 10\(^{-3}\) M chromate or saline. The platelets were sedimented, resuspended in 0.1 per cent EDTA in saline and resedimented. The platelet button from 5 ml of PRP was extracted with 3 ml of 1N perchloric acid and the O.D. of the extract measured at 258 μ.

Serotonin release was studied by using platelets labeled with \(^{14}\)C Serotonin (Nuclear Chicago, Sp. A. = 30 mCi./mM). Citrated PRP was incubated with labeled serotonin (0.08 μCi./ml PRP) for 30 minutes at 37°C. Sodium chromate in saline was added to one half the specimen, and an equal volume of saline was added to the other half. Incubation was continued at 37°C for 75 minutes. One-ml samples of the PRP were placed in plastic tubes, to which were added 0.1 ml of either dilute CT or IBS. The tubes were capped and tumbled on a vertical rotating turntable for six minutes at room temperature. Equal volumes of the tumbled PRP or supernatant PPP were added to 15 ml of liquid scintillator and their radioactivity was measured in a Nuclear Chicago liquid scintillation counter. The release of serotonin (CPM supernatant/CPM total) × (100) caused by CT, was adjusted by subtracting the per cent of radioactivity present in the control supernatant.

Platelet factor III activation was tested by the method of Zucker and Peterson.\(^7\) PRP was incubated with chromate or saline, and placed in the aggregometer. At 0, two and five minutes after the addition of dilute CT, 0.1-ml aliquots of PRP were removed from the cuvette and were immediately pipetted into test tubes containing 0.1 ml of 0.025M...
calcium chloride, and 0.1 ml. of Russell viper venom at a concentration of 10 μGm./ml. in IBS. Clotting times of these mixtures were measured.

The amount of chromium bound to platelets under the present conditions was measured by a radioactive tracer technique. Sodium chromate was added to 100 ml. quantities of PRP at a final concentration of either 10^{-4}M or 10^{-5}M along with 25 or 50 μCi. of ^{51}Cr sodium chromate (Abbott, specific activity: 100 mCi./mg.). The final concentration of chromate was not significantly affected by the labeled chromate. The mixture was incubated for 90 minutes at 37°C, after which 1-ml. amounts were removed and set aside for counting. Ten ml. of one per cent EDTA in saline were added and the platelets were sedimented in a clinical centrifuge at 2500 RPM for 30 minutes. They were resuspended in 0.1 per cent EDTA in saline, and this washing was repeated until the radioactivity of the wash fluid was stable at about 50 CPM above background (usually by the fifth wash). The platelets were resuspended in 4 ml. of the wash solution, and the radioactivity of the platelet suspensions as well as that of the original PRP was measured in a Nuclear Chicago well-type scintillation counter. The amount of chromium bound to platelets was derived from the following expression:

\[
\text{Moles Cr/10^9 platelets} = \frac{\text{CPM/10^9 platelets}}{\text{CPM of orig. PRP}} \times \text{initial Cr concentration.}
\]

**RESULTS**

**Connective tissue induced aggregation.** Sodium chromate markedly inhibited connective tissue induced platelet aggregation (Fig. 1). This effect increased with time of incubation and was greater at an incubation temperature of 37°C than at 25°C. The lowest concentration of chromate which would inhibit CT-induced platelet aggregation was 10^{-5}M; at this concentration, inhibition was minimal.

Inhibition was specific for hexavalent chromium. Trivalent chromium in the form of chromic chloride (10^{-2}M) when incubated with PRP for one hour at 37°C did not affect CT-induced aggregation. Furthermore, the oxidant potential of chromate is not responsible for the altered platelet response. Potassium permanganate, a stronger oxidizing agent than chromate, did not affect platelet aggregation by CT when incubated at a concentration of 10^{-4}M with PRP for one hour at 37°C.

Chromium affects the platelet, not CT. CT was incubated with sodium chromate (0.1M) for one hour at 37°C, and was washed three times. The chromium-treated CT aggregated platelets as well as an equal amount of similarly incubated and washed nonchromated CT (Fig. 2).

![Fig. 1.—Inhibition of connective tissue-induced platelet aggregation, by sodium chromate (10^{-4}M). Incubation time represents duration of chromium exposure.](image-url)
**ADP, thrombin, and epinephrine-induced aggregation.** Since CT-induced aggregation is mediated by ADP, the effect of chromium upon ADP-induced platelet aggregation was studied. ADP-induced aggregation was inhibited by only 20 per cent after 80 minutes of incubation with $10^{-4} M$ chromate at $37^\circ C$. Comparison of the slope of inhibition of ADP-induced aggregation with that of CT-induced aggregation reveals a clear divergence (Fig. 3), implying that the effect of chromate upon CT-induced aggregation is not mediated primarily through an effect on ADP.

Thrombin-induced aggregation, which also may be mediated by ADP, was unaffected by chromate, although minimal amounts of thrombin (0.1–0.2 units/ml. final concentration) were used, and the incubation of PRP with chromate ($10^{-4} M$) was carried to 90 minutes at $37^\circ C$.

Chromate inhibited the primary wave of epinephrine-induced platelet aggregation (Fig. 4) by 60 per cent when PRP was incubated with $10^{-4} M$ chromate for 80 minutes at $37^\circ C$. The secondary wave of platelet aggregation normally caused by epinephrine was unaffected until the primary wave had been significantly inhibited.

**Platelet adhesion to connective tissue.** Since chromium inhibited CT-induced platelet aggregation without a significant effect upon ADP action, the possibility was explored that platelet–CT adhesion might be the site of the chromium effect. This appeared not to be the case, as demonstrated by the
Effect of chromium on platelet function in vitro

Table 1.—Supernate Optical Density/10^6 Platelets/mm^3

<table>
<thead>
<tr>
<th>Control</th>
<th>Chromate</th>
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<tbody>
<tr>
<td>1 0.94</td>
<td>0.70</td>
</tr>
<tr>
<td>2 0.88</td>
<td>0.28</td>
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<tr>
<td>3 2.90</td>
<td>1.20</td>
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Table 2.—Per cent Release of ^14C Serotonin

<table>
<thead>
<tr>
<th>Control</th>
<th>Chromate</th>
</tr>
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<tbody>
<tr>
<td>1 39</td>
<td>10</td>
</tr>
<tr>
<td>2 56</td>
<td>17</td>
</tr>
<tr>
<td>3 30</td>
<td>8</td>
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Normal O.D. response of chromated platelets to concentrated CT in the presence of EDTA, a specific measure of adhesion.

Connective tissue-induced release reaction. Since adhesion appeared unaffected by chromate, the next phase of CT-platelet interaction, the "release" reaction, was studied. The effect of chromate on CT-induced release of adenine nucleotides, labeled serotonin and activation of platelet factor III were examined.

Chromate inhibited the release of platelet adenine nucleotides normally caused by CT. After incubation of EDTA PRP with chromate (10^{-3}M) for 90 minutes at 37°C, the platelets were washed, resuspended and tumbled with concentrated CT. The O.D. at 258 m\(\mu\) was lower in the supernates prepared from chromated platelets, as compared to those from control platelets (Table 1). This result was evidently not due to depletion of adenine nucleotides, since total platelet adenine nucleotide content was unaffected by incubation with chromate.

Chromate also greatly inhibited the CT-induced release of ^14C serotonin from platelets labeled in vitro with this compound, as shown with labeled
PRP which had been incubated with $10^{-3} M$ chromate for 75 minutes at 37°C (Table 2).

CT-induced platelet factor III activation as measured by the shortening of the RVV time of PRP, was likewise inhibited by chromate. When citrated PRP was incubated with chromate ($10^{-3} M$) at 37°C, the inhibition of PF3 activation paralleled the inhibition of aggregation; with increasing time of incubation with chromate, PF3 activation decreased (Table 3).

**Amount of chromium bound to platelet.** The in vitro aggregation of platelets which have been processed by the method normally used to label them for survival studies (i.e., acidification to pH 6.5, sedimentation and resuspension), is significantly suppressed, even if the pH of the resuspended PRP is returned to 7.5. Consequently, it was difficult to evaluate the effect of chromate on platelets handled in this manner. However, it was possible to assess the effect of chromium labeling on platelet function by measuring the amount of chromium which must be bound to platelets to inhibit their function. This figure could then be compared to values reported for the amount of chromium bound to platelets when they are labeled for survival studies. Tracer amounts of $^{51}$Cr chromate mixed with nonradioactive chromate were added to PRP and the mixtures were incubated for 90 minutes at 37°C. In two experiments using a final concentration of chromate of $10^{-4} M$, the amount of chromium remaining in the washed platelets was 3.2 and $8 \times 10^{-9}$ moles/10$^9$ platelets, respectively (Table 4). In one experiment using $10^{-5} M$ chromate, the uptake of chromium was $1.5 \times 10^{-9}$ moles/10$^9$ platelets.

| Table 3.—Effect of $10^{-3} M$ chromate on connective tissue-induced decrease in Russell Viper venom time at two and five minutes of ACT added compared with its effect on aggregation. Two separate samples of PRP were tested. |
|---|---|---|
| **Per Cent Inhibition of Aggregation** | **Per Cent Decrease in RVV Time**<br>**Incubation Time with CT** |
| **Experiment #1** | **Two Minutes** | **Five Minutes** |
| Control | 19 | 38 |
| Chromate | 6 | 28 |
| **Experiment #2** | | |
| Control | 8 | 33 |
| Chromate | 6 | 8 |

* (Control clotting time) – (CT-induced clotting time) × 100

| Table 4.—Platelet uptake of “cold” chromium after incubation for 90 minutes at 37°C in the presence of tracer amounts of radioactive chromium. Three separate experiments are given. |
|---|---|---|---|
| **Total Ambient Cr Content** | **CPM of PRP × 10^6** | **CPM/10^9 Washed Platelets × 10^9** | **Moles Cr/10^9 Platelets** |
| 1 | $10^{-4} M$ | 494 | 4.14 | $8 \times 10^{-9}$ |
| 2 | $10^{-4} M$ | 498 | 1.63 | $3.2 \times 10^{-9}$ |
| 3 | $10^{-5} M$ | 237 | 3.67 | $1.5 \times 10^{-9}$ |
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DISCUSSION

The present studies indicate that hexavalent chromium (chromate) has an adverse effect on platelet function in vitro. The major effect was the inhibition of connective tissue-induced platelet aggregation. ADP-induced aggregation was only slightly inhibited, and thrombin-induced aggregation was unaffected. The primary wave of epinephrine-induced aggregation was significantly inhibited; the secondary wave was affected only after supression of the primary wave.

The initial reaction between platelets and connective tissue in vitro and in vivo, is adhesion. This is followed by the "release reaction" in which intraplatelet ADP, serotonin and other compounds emerge, and platelet factor III is activated. The released ADP causes the aggregation that ensues. Chromium did not sufficiently inhibit the platelet response to ADP to account for its effect on CT-induced aggregation; nor did chromium reduce the adhesion of platelets to connective tissue. The data thus indicate that chromium inhibits CT-induced aggregation by inhibiting the "release reaction" normally caused by CT and thereby prevents the release of platelet ADP necessary for aggregation.

Because the mechanism of the primary wave of epinephrine-induced aggregation is unknown, the basis of the chromium effect on this function is uncertain. The secondary wave of aggregation, which depends on the release of intraplatelet ADP, is similar to CT induced aggregation and one might expect that this would be inhibited by chromium. However, the inhibition of the primary wave prevented any meaningful observations of the secondary wave.

The biologic activity of chromium has recently been extensively reviewed. It is apparent that the active form of chromium, which binds to organic substrates such as protein, is trivalent. Hexavalent chromium, the form used in labeling, is active only after it has been reduced to the trivalent state. Studies in red cells, skin and other living cells have shown that only hexavalent chromium, by not binding to biologic material, can enter cells. During this entry, it is reduced to the trivalent form and is captured by the cell as it binds to intracellular substrates.

The site of chromium activity in platelets is probably intracellular for the following reasons: 1) only the hexavalent form of chromium, chromate, inhibited platelet function; 2) the oxidant properties of chromate are not responsible for its effect, since permanganate, a stronger oxidant than chromate, did not affect platelet function; 3) chromate inhibition of platelet aggregation increased with time of incubation, and increased when the temperature of incubation was raised from 25 to 37°C. This conforms with the demonstration by Davey that platelet uptake of chromium increases with time of incubation and is maximal at 37°C. Not only does this latter observation indicate that the site of chromium activity is within the platelet, but also that the degree of inhibition is probably related to the amount of chromium that enters the platelet.

The precise intracellular site at which chromium acts is unknown. A recent report on the intraplatelet sites of 51Cr binding indicates that chromium is bound to thrombosthenin and at least one other unidentified protein, as well
as mitochondria, granules and cytoplasmic ADP. Chromium, in excessive amounts, can inhibit enzymatic function, and its presence at any of these sites of binding could interfere with normal metabolic activity. The complexing of chromium with ADP would not account for the inhibition of platelet function caused by chromium. When ADP was incubated with trivalent chromium there was no loss of its aggregating activity.

Whether chromium has a corresponding effect on the in vivo function of platelets labeled with this agent is not clear. The amount of chromium which must be bound to platelets to inhibit their function is 10–100 times greater than that bound under the usual conditions of labeling. Aster, in discussing his method, estimated that 10 per cent of the chromium used for labeling was bound. This yields a figure of $0.67 \times 10^{10}$ moles Cr/10$^9$ platelets. A comparable figure for Davey is $5 \times 10^{10}$ moles Cr/10$^9$ platelets. We have used $5.7 \times 10^9$ moles Cr/10$^9$ platelets as the amount bound at that concentration (10$^{-4}$M) which causes significant inhibition of platelet function. However, our studies of platelet function were limited by the time of incubation, for even nonchromated platelets deteriorate in vitro. Since the chromium binding is essentially permanent, it may be that the smaller amount used in labeling creates a lesion which eventually interferes with platelet function, but becomes manifest only after several hours in vivo. It is also possible that small amounts of chromium cause a physiologic lesion which is undetectable in vitro by our present means of assessing platelet function.

If labeling with radioactive chromate does affect platelets it might not alter platelet viability. For example, it has been shown that the treatment of platelets with agents that permanently suppress the CT-induced release reaction may actually prolong survival. Therefore, the normal life span of chromated platelets does not perforce indicate normal function.

That platelets labeled with chromate do not function normally is suggested by the observation that most platelet survival curves performed with labels other than radioactive chromate demonstrate a degree of random loss which perhaps indicates participation of platelets in a hemostatic reaction. However, in survival studies performed with radioactive chromate, this random loss component is generally absent, suggesting that these platelets have reduced hemostatic properties. Since the primary hemostatic step is platelet interaction with connective tissue, the absence of random loss in chromated platelets may be explained by the effect of chromate in inhibiting this function. Accordingly, the interpretation of the kinetics of survival curves performed with $^{51}$Cr chromate labeled platelets may be misleading.

**SUMMARY**

Sodium chromate inhibits platelet function in vitro. The primary effect is inhibition of connective tissue-induced aggregation. In addition, the primary wave of epinephrine-induced aggregation is moderately inhibited and adenosine diphosphate-induced aggregation is mildly inhibited. The effect on connective tissue-induced aggregation is due to inhibition of the platelet “release reaction”; chromate inhibited the release of adenine nucleotides, $^{14}$C labeled serotonin and the activation of platelet factor III normally caused by
connective tissue. The amount of chromium which must be bound to platelets to inhibit aggregation is 10–100 times the amount of radioactive chromium bound to platelets under the usual conditions of labeling for survival studies. However, this does not imply that chromium labeled platelets function normally.

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REFERENCES

The Effect of Chromium on Platelet Function In Vitro

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